PREPARATION AND CHARACTERIZATION OF EXTRACELLULAR VESICLES FOR CANCER THERAPY

Heikki Saari

Department of Pharmaceutical Biosciences and Center for Drug Research

Faculty of Pharmacy

University of Helsinki

Finland

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Supervisors

Professor Marjo Yliperttula
Division of Pharmaceutical Biosciences and Drug Research Program
Faculty of Pharmacy
University of Helsinki Finland

Pia Siljander
Division of Pharmaceutical Biosciences and Drug Research Program
Faculty of Pharmacy
EV-core unit, Molecular and Integrative Biosciences Research Program
Faculty of Biological and Environmental Sciences
University of Helsinki Finland

Tapani Viitala
Division of Pharmaceutical Biosciences and Drug Research Program
Faculty of Pharmacy
University of Helsinki Finland

Pre-examiners

Samir El-Andaloussi
Department of Laboratory Medicine, Clinical Research Center
Karolinska Institutet

Tuija Kekäräinen
Kuopio Center for Gene and Cell Therapy

Opponent

Pieter Vader
Laboratory of Clinical Chemistry and Haematology & Department of Experimental Cardiology
University Medical Center Utrecht

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Abstract

Drug delivery aims to optimize the systemic distribution of administered drugs to reach their target tissue in an effective and specific manner. This approach is arising also in the field of cancer therapy, since traditional small molecule chemotherapeutics can cause severe side effects and more sophisticated biomolecular therapeutics cannot always reach the target cells on their own. For these purposes, therapeutics can be packed into carriers that improve their pharmacokinetics by providing a suitable, protected environment for the cargo to travel in the body, armed with targeting molecules that guide them to the site of interest. Synthetic drug carriers include liposomes, porous silica and different polymer particles that can be modified to contain necessary surface structures for targeted delivery and improved biocompatibility. While they have shown much promise in research settings, their success in cancer treatment has been limited to only a handful of commercially available formulations, some of which have presented liver toxicity as a result of continuous administration. Additionally, targeting cancer cells is difficult because cancer is a very individual disease, and heterogeneity exists even within tumors, so finding any universal cancer-specific markers to target is extremely challenging.

Nature has adopted a similar approach for delivering molecules containing biologically functional cargo from cell to cell. These naturally occurring carriers are called extracellular vesicles (EVs), in contrast to intracellular vesicles that mediate cargo trafficking inside of the cells. EVs are 50 – 1000 nm in diameter, consisting of a lipid bilayer with embedded membrane proteins that encloses water-soluble biological cargo within. Their exact composition and cargo vary from cell to cell and depending on the condition of the cells that produce them. Additionally, cells have multiple pathways for EV secretion and production. For these reasons EVs comprise a very heterogeneous population of vesicles in composition and they also have multiple biological functions that have not been completely clarified yet. EVs have been found in practically all body fluids and organisms that have been studied, taking part in the normal and pathological functions of the organism. For example, procoagulant EVs are found in blood and saliva that participate in hemostasis, while EVs secreted by cancer cells promote the survival, growth and metastasis of the tumor. They are able to affect cells via membrane interactions and by delivering functional cargo into their recipient cells. It has been shown that EVs are even able to target cells selectively by binding to specific cellular receptors, enabling targeted cargo delivery.

Given their natural cargo delivery properties, EVs present remarkable potential for drug delivery applications. As cancer cells also use EVs to communicate with each other, their EVs reflect the same heterogeneity that exists within the cells themselves, and may provide useful insights for cancer-targeted drug delivery. However, the internalization mechanisms of drug-carrying EVs and especially the fate of the drugs they carry is not well understood. In this thesis, the use of EVs was assessed for the delivery of chemotherapeutic drug paclitaxel and an oncolytic adenovirus that represent small molecular and biological chemotherapeutics, with cancer cell-derived EVs as the model carrier. The studies presented here focus on the preparation, characterization, intracellular tracking and effectiveness of these EVs for cancer therapy. The obtained results provide novel methods and knowledge for the future development of EV-based therapeutics for the treatment of cancer. First, EVs were loaded with paclitaxel by incubation, which enhanced the cytotoxic effect of the drug, changing its internalization from passive diffusion to endocytosis. A novel approach using fluorescence lifetime microscopy was introduced for tracking the release of paclitaxel from the EVs inside of the cells, identifying distinct patterns of subcellular drug release in individual cells and its intracellular kinetics. This method was able to show details about the drug release mediated by EVs that could not be observed with conventional fluorescence microscopy. Additionally, the relationship between EVs and adenoviruses was explored, revealing a previously undocumented pathway of spreading adenoviral infection via EVs. This EV-mediated infective delivery of the viral genome occurred separately from the classical pathway of adenoviral life cycle, and produced infective particles resembling EVs more than virions. EVs can in theory enhance the pharmacokinetics of oncolytic viruses by hiding them from the immune system and providing them alternative pathways for cell targeting and internalization, however it was found that infective EVs do react with adenovirus neutralizing antibodies. Taken together, these results suggest that EVs can act as versatile carriers of therapeutic cargo for cancer treatment, ranging from small molecule chemotherapeutics to oncolytic viruses, though they require further development. New methods are reported constantly for preparing and studying therapeutic EVs with new, innovative approaches that will help in the future treatments of cancer and other diseases.

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As a biophysicist, Tapani's field of research is quite different from mine, but he has always been eager to help in my projects when I needed his opinion or assist with practical issues, showing genuine interest in my work and willingness to help. Tapani has also shown great trust in my expertise, linking me to his projects related to EVs and thus created valuable networking and visibility for me. Pia is an experienced EV-researcher and she has been a great mentor to me in that regard already before starting this work as a supervisor of my master's thesis. She has very high standards, which is especially important in a developing field such as the EV field, and it has also greatly affected my work, elevating its level. We have had many great discussions about science, and no one has given me as much feedback on my writings as she has. I deeply appreciate Pia's honesty, straightforwardness and encouragement; those are such important qualities for a good supervisor such as her. Finally, my primary supervisor Marjo, is who this all started with. She has shown unprecedented faith in me during the time I have worked as a part of her team, letting me work independently and even giving me supervision over EV-research in her Biopharmacy group. Marjo has had a significant role in raising young scientists such as myself, making sure they have as bright a future as possible. This appears to be an unfortunately rare quality in academia, but her skill to guiding is where she truly excels as a leader. She is also very open to new ideas and trying new things, which I have been able to relate with, and she has an almost eerie ability to always find a way to make things happen, allowing creativity to flourish. Thank you all three for being my supervisors during this time.

I have the great honor of having doctor Pieter Vader from the University Medical Center Utrecht as my opponent. I am grateful that he took the time for me, due to the current situation with the COVID-19 pandemic we will unfortunately not be able to meet in person for the dissertation, but I am certain we will see in the future. I would also like to thank doctor Samir El-Andaloussi from Karolinska Institute, Stockholm and professor Tuija Kekäräinen from Kuopio Center for Gene and Cell Therapy for being my pre-examiners and professor Kari Keinänen and doctor Marc Baumann for serving as members of my thesis committee, all giving me valuable feedback during my thesis work.

I would also like to thank all of the co-authors, who I have worked with on the present studies. When I started working on EVs in Marjo's group, I was teamed up with Elisa Lázaro-Ibáñez. We didn't really know, what we were doing at first and didn't agree on many things, but we made a good team anyways and it was fun working and travelling with you. You are a great person and always worked incredibly hard, it was borderline insanity. Ekaterina Lisitsyna is my favorite

spectroscopist, although I never really saw you measure any spectras. You have great critical thinking capability, which will take you far with EVs and other things. I also have never seen anyone eating raw lemons so casually, maybe you would be too sweet otherwise. Andres Lõhmus I already knew before recruiting you to Marjo's team, because we made a connection through dad jokes on a lab course and I taught you to lift things. I always appreciated our stupid conversations and your endless knowledge. You are like a walking encyclopedia, which is convenient since I don't even need google with you. You should have more faith in yourself though, comparing yourself to me will only lead in disappointment, hopefully this book will inspire you to greatness. Thanks also to Tatu Rojalin, you were great company in Toronto and your enthusiasm in science always inspired me. To all of the other authors: Elina Vuorimaa-Laukkanen, Kaisa Rautaniemi, Liisa Niemi, Olli-Petteri Nivaro, Timo Laaksonen, Tiia Turunen, Mikko Turunen, Matti Jalasvuori, Sarah Butcher, Seppo Ylä-Herttuala and Vincenzo Cerullo, it has been a real pleasure working with you all.

While working at the university, I was never too sociable, and although I don't really express it I have always had great co-workers. With the Biopharmacy group we have had many great trips and more importantly many great games of BANG!, Saboteur and others. I wish we played more often, because you are all fun to play with, but you were also great to work with, being helpful and considerate. So thank you Raili, Patrick, Sami, Riina, Vili, Jacopo, Arto, Jasmi, Mariangela, Petter, Laura A, Elina and Laura M for your company. I also have worked a lot with the EV-group, we had lots of good discussions about EVs and good times, and for some reason you all had very similar names: Mari, Maria, Maarit and Katri-Maarit. Thanks for your company and may you work with similarly named people in the future as well. Also I thank Leena Pietilä and Timo Oksanen for keeping the labs running and making our work possible, and the ImmunoViroTherapy group for their assistance in my virus works. Thank you to all the other workers at the faculty of Pharmacy for such a nice working environment, especially Jaakko Itkonen for the best bouldering company and commute (sigh). I thank my family and friends for their support and shared time through all these years, especially my girlfriend Hanna, who has tolerated me for so many years.

Finally, I would like to dedicate this work to the memory of my friend Lari Häkkinen. I miss your kindness and good spirit. I wish that this work will help bringing hope to all people with cancer.

List of original publications

This thesis is based on the following publications:

I Saari, H., Lázaro-Ibáñez, E., Viitala, T., Vuorimaa-Laukkanen, E., Siljander, P., & Yliperttula, M. (2015). ”Microvesicle-and exosome-mediated drug delivery enhances the cytotoxicity of Paclitaxel in autologous prostate cancer cells”, Journal of Controlled Release, 220, 727-737.

II Saari, H., E. Lisitsyna, K. Rautaniemi, T. Rojalin, L. Niemi, O. Nivaro, T. Laaksonen, M. Yliperttula, and E. Vuorimaa-Laukkanen. "FLIM reveals alternative EV-mediated cellular up-take pathways of paclitaxel", Journal of controlled release (2018).

III Heikki Saari, Tiia Turunen, Mikko Turunen, Matti Jalasvuori, Sarah Butcher, Seppo Ylä-Herttuala, Tapani Viitala, Vincenzo Cerullo, Pia Siljander, Marjo Yliperttula. ”Extracellular vesicles provide a capsid-free vector for oncolytic adenoviral DNA delivery”, Journal of Extracellular Vesicles (submitted in 2019, accepted in 2020).

The publications are referred to in the text by their roman numerals.

Author's contribution

Publication I

The author performed background research of existing literature and participated in planning the experiments, especially those regarding cytotoxicity and drug loading of extracellular vesicles (EVs) and their imaging with live-cell microscopy. The author produced and assessed EVs for these experiments, and others including cryo-electron transmission microscopy and zeta-potential measurements, and performed the cytotoxicity measurements. The author participated in writing of the manuscript and data analysis, especially the portions regarding drug delivery with EVs and their statistical analysis.

Publication II

The author designed and performed extracellular vesicle production, isolation and characterization, and co-designed, supervised and analyzed confocal microscopy experiments. The author also participated in interpreting fluorescence lifetime imaging results and writing of the manuscript.

Publication III

The author performed vast majority of study design, experimental work, data analysis and writing. Co-authors participated in designing individual experiments and PCR-primers, performed qPCR-analysis and sample preparation for transmission electron microscopy, took part in data interpretation and gave feedback during writing of the manuscript.

Table of Contents

1 Introduction

1.1 Extracellular vesicles

Extracellular vesicles (EVs) are submicron sized membrane particles that all cells secrete into their surroundings. While their physiological relevance is not completely understood yet, they have been shown to mediate cell-to-cell communication, between both short and long distances within an organism. Given their large size compared to other intercellular messengers, such as hormones, the structure and composition of EVs is very complex (Figure 1). They are also affected by the type of the EV secreting cell and its status, which are then both reflected on the EVs and their function. Furthermore, several cellular pathways for EV generation have been identified, adding even more to their heterogeneity in the form of EV subtypes. Taken together, these qualities make EVs an extremely versatile and flexible intercellular communication system. While different cells of all organisms have been shown to secrete EVs, here we will focus on the EVs of mammalian cells, as they are the most relevant regarding therapeutic or drug delivery applications, with an emphasis on the role of EVs in the progression of cancer.

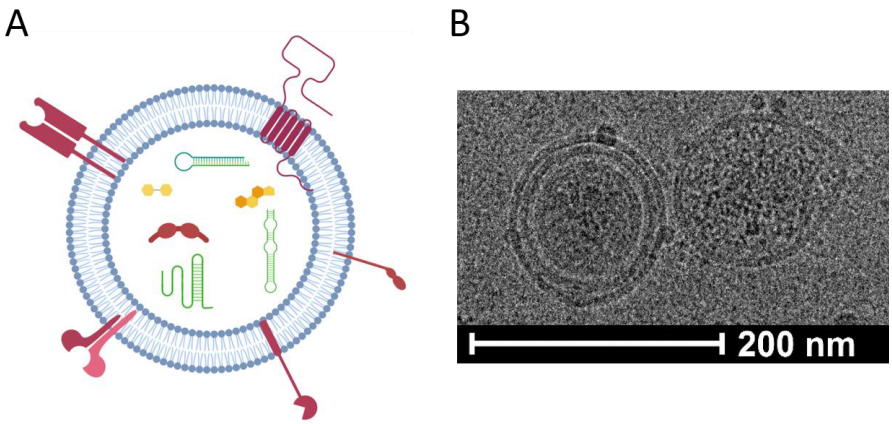


Figure 1: Structure of EVs. A: Schematic representation of EV structure. EVs are composed of a membrane that contains lipids (blue) and membrane proteins (red) that encloses intraluminal contents, including soluble proteins, nucleic acids (green) and metabolites (yellow). B: Cryo-electron micrograph of EVs. A picture of two PC-3 EVs from study (III) that was acquired using cryo-transmission electron microscopy. While the sizes of the EVs are similar, the EV on the left contains three membranes, while the one on the right only has one membrane.

1.1.1 Biogenesis of extracellular vesicles

Several biogenesis pathways have been identified for EVs, which is one way to classify EV subtypes (van Niel, D'Angelo & Raposo, 2018). The coexistence of parallel pathways for EV biogenesis is one of the major factors contributing to the observed heterogeneity in the isolated EV populations. Three of the most commonly described subtypes are i) exosomes, ii) microvesicles and iii) apoptotic bodies, all with distinct mechanisms of generation that may vary in their details depending on the cell type. ***Apoptotic bodies*** are a type of EVs with a widely varying diameter from approximately 50 nm up to several micrometers that bud from cells undergoing programmed cell death and thus differ fundamentally from the other EV subtypes. ***Exosomes*** are formed in the endosomal network of the cell by inward budding of late endosomes. This creates intraluminal vesicles inside the endosome, turning it into a multivesicular body that can eventually fuse with the plasma membrane releasing the intraluminal vesicles, now termed exosomes. ***Microvesicles***, on the other hand, are formed at the plasma membrane by a similar budding process, with the generated vesicles getting released straight into the extracellular space. The budding of both exosomes and microvesicles both require the accumulation of specific lipid species and proteins associated with the budding process and cargo sorting at the vesicle budding site (van Niel, D'Angelo & Raposo, 2018).

Some of the most important proteins involved in the EV formation and cargo loading are those of the endosomal sorting complex required for transport (ESCRT), which mediate the clustering of lipid and protein cargo and structural elements and the following vesicle budding event as well (Colombo *et al*., 2013). Four ESCRT complexes have been identified, which form the machinery for budding and cargo sorting of intraluminal vesicles forming in multivesicular bodies (Christ *et al*., 2017): ESCRT-0 binds monoubiquitinated membrane proteins and phosphatidylinositol-3-phosphate at the budding site, causing their accumulation by forming multiprotein scaffolds with clathrin. ESCRT-I then binds ESCRT-0, in turn recruiting ESCRT-III with either ESCRT-II or Alix. ESCRT-III then mediates scission of the forming vesicle with VPS4(Adell *et al*., 2014). Exosomes can also be formed through ESCRT-independent pathways, involving a protein complex formed by syndecan, syntenin and Alix, the accumulation of ceramide or proteins of the tetraspanin family, especially CD63 (Trajkovic *et al*., 2008, Edgar, Eden & Futter, 2014). These pathways induce membrane curvature and clustering of cargo at the budding sites in multivesicular bodies, followed by detaching of the vesicle from the membrane. Several post-

translational modifications have been found enriched in EV-associated cargo proteins, including ubiquitination, SUMOylation, phosphorylation, specific glycosylation patterns and more, indicating that cargo sorting into EVs is at least partly regulated by these modifications, which may be bound by ESCRT proteins, tetraspanins or ceramide during vesicle formation (Anand *et al*., 2019). While there are similarities between the budding process of microvesicles and exosomes, the budding of microvesicles is dependent on the asymmetric accumulation of lipids such as phosphatidylserine (PS) and cytoskeleton reorganization at the budding site, because for membrane budding to occur at the plasma membrane, cytoskeleton-plasma membrane connections must be flexible enough to allow localized membrane curvature. These events are mediated by Ca^{2+}-dependent lipid translocases, calpain or protein kinase C (Gonzalez *et al*., 2009, Stratton *et al*., 2015, Taylor, Jaiswal & Bebawy, 2017), and they are involved in the formation of the initial curvature of the budding membrane (Piccin, Murphy & Smith, 2007, Sidhu *et al*., 2004). Proteins of the ESCRT complex, such as TSG101 are recruited to the plasma membrane from the endosomes to mediate the budding and cargo sorting events of microvesicles (Nabhan *et al*., 2012). The final scission is usually executed by the contraction of actin and myosin, induced by GTP-binding proteins such as ADP ribosylation factors 6 and 1 (ARF6 and ARF1) (Muralidharan-Chari *et al*., 2009), ESCRT-III-VPS4(Nabhan *et al*., 2012) or rho-associated, coiled coil containing protein kinases (ROCK) (Li *et al*., 2012).

1.1.2 Composition of extracellular vesicles

The biogenesis of EVs involves the accumulation of lipids such as PS and ceramide to the budding site along with proteins of the ESCRT machinery or alternative proteins, which can selectively transport intended cargo molecules into the forming vesicles and then remain part of the EV structure when it detaches. Consequently, while EVs contain virtually all of the major types of biomolecules including lipids, proteins, nucleic acids and metabolites, the amount of these molecules in EVs may differ significantly compared to the cells they are derived from. Regarding the lipids that form the membrane structure, certain species are concentrated in EVs most likely to support the strong membrane curvature that is required due to their small size (Figure 2). Lipids such as cholesterol, PS, phosphatidylinositol (PI), phosphatidylethanolamine and ceramide cause

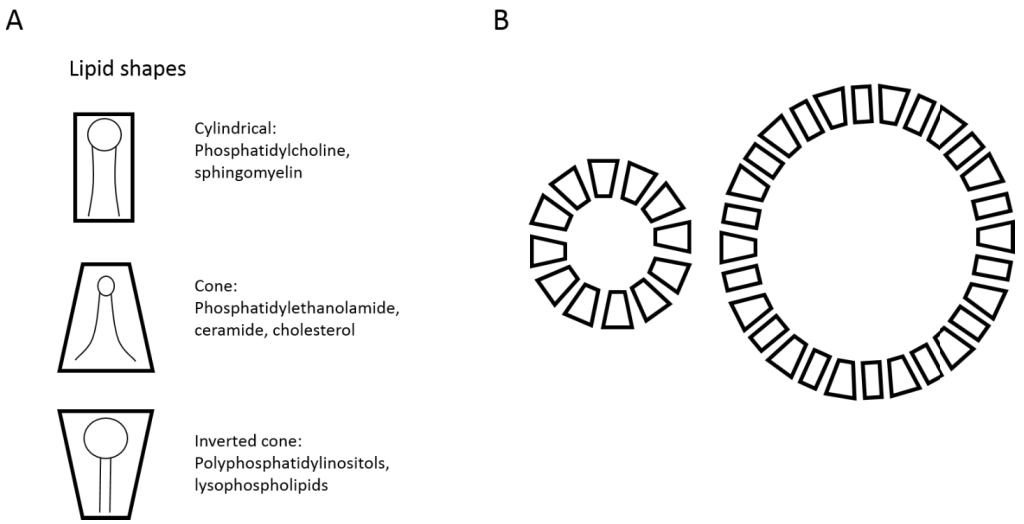


Figure 2: Schematic representation of lipid shapes and their effect on membrane curvature. A: Classification of lipids according to their approximate geometrical shape with examples of each group. The size of the lipid head group compared to their tail determines their overall shape, with cylindrical lipids having equal sized head and tail, while in conical lipids the differences in group size cause an irregular overall shape. B: The effect of lipid composition on membrane curvature. A relatively small membrane structure (left), such as those found in EVs, has stronger membrane curvature, leaving less room for cylindrical lipids. A larger membrane structure (right), such as those found in cells, requires more cylindrical lipids to reduce membrane curvature.

either positive or negative curvature due to the relative size difference between their head and tail groups, which gives them a conical shape, while cylindrical lipids such as phosphatidylcholine (PC) have equal sized head and tail (Janmey, Kinnunen, 2006). EVs have been found to contain more conically shaped lipids and less cylindrical lipids, when compared to larger membrane structures such as cellular organelles (Llorente *et al*., 2013, Haraszti *et al*., 2016). EVs are especially enriched in cholesterol, which provides also membrane rigidity, while the amount of phospholipids is diminished as a result (Llorente *et al*., 2013, Brzozowski *et al*., 2018). Also, the lipids in EVs appear to be more saturated. While tail length and saturation level of phospholipids also affect their shape, the head group appears to be the most important determining factor for enrichment in EVs, as head group distribution in EVs contains more variation compared to the

cells' lipid profile than tail length and saturation. The phospholipid composition found in EVs includes less phosphatidylcholine and more PS, which is required in the initial phases of microvesicle budding. Other, less abundant phospholipids including PI and phosphatidylglycerol are more enriched in cells than EVs and the amount of glycerolipids is approximately the same in both cells and EVs. While sphingolipids are also enriched in EVs compared to cells, sphingomyelin and its derivatives have a cylindrical structure (Janmey, Kinnunen, 2006, Goñi, Alonso, 2006). Therefore, the role of sphingomyelins cannot be explained with supporting membrane curvature, i.e. they could be replaced by much more prevalent PC. Hence sphingomyelins must have a specific, functional role. Indeed, sphingolipids are considered to be the most complex lipids of all, because they are also glycosylated and they have been found to be involved in cell recognition and signaling (Hannun, Obeid, 2008). Sphingolipids can also form lipid raft-like structures on EVs that act as scaffolding sites for certain membrane proteins, given that lipid rafts provide another alternate mechanism for EV cargo sorting (Gassart *et al*., 2015). As with cellular membranes, the lipids are asymmetrically distributed in the EV membrane into the outer and inner leaflets (Llorente *et al*., 2013). While the lipid distribution has not been extensively studied, EVs display PS on their outer leaflet, which is considered a marker for apoptotic cells and acts as an "eat me" signal to phagocytes, or in the case of activated platelets, exposed PS promotes coagulation (Frey, Gaipl, 2011, Segawa, Nagata, 2015).

Proteomic meta-analyses indicate that most common cellular functions of EV-associated proteins are related to signaling, transport events and cell motion (György *et al*., 2011, Choi *et al*., 2013). Additionally, the exact proteome of EVs varies heavily depending on the secreting cell, while their lipid composition appears to be relatively unchanged across different cell types (Haraszti *et al*., 2016, Brzozowski *et al*., 2018, Choi *et al*., 2013). The most commonly found EV-enriched proteins include tetraspanins such as CD9, CD63 and CD81, integrins, heat shock proteins, Alix, TSG101 and others that are associated with EV biogenesis or cargo sorting. These proteins are, therefore, commonly used as markers for EVs in research (Colombo, Raposo & Théry, 2014). The EV-associated proteins have also been found to contain specific glycosylation patterns that vary between cell types and may act as protein sorting signals during EV biogenesis or in target recognition after secretion (Batista *et al*., 2011, Saraswat *et al*., 2014, Costa, 2017). More specifically, EVs from different cell types were all found to be enriched in polylactoseamine, α2-6 linked sialic acid, high mannose and complex type glycans, while blood group antigens A and B

were not found in EVs. Because proteins have been shown to be able to retain their activities on EVs, they can together form functional units, with the EVs serving as scaffolds bringing them together. For example, cancer cells have been shown to secrete EVs that modify distant sites to develop so called metastatic niches, where circulating cancer cells can attach and grow metastases (Hood, Roman & Wickline, 2011, Hoshino *et al*., 2015). This can be accomplished via combining target recognition with appropriate cargo delivery, signaling via cell membrane receptors and even enzymatic activities that EVs may carry, such as matrix metalloproteases and other enzymes that cleave extracellular matrix in the target region to ease cancer cell attachment and growth (Nawaz *et al*., 2018).

Arguably, the most interesting cargo type identified in EVs so far is the nucleic acids. This interest is due to the observation that the nucleic acid content of EVs is able to affect the gene expression of the recipient cell as a type of horizontal gene transfer (Ratajczak *et al*., 2006, Waldenström *et al*., 2012, Cai *et al*., 2013, Cai *et al*., 2014, Wei *et al*., 2017, Hinger *et al*., 2018), thus having long lasting, profound effects on its function. These nucleic acid species include DNA and different types of RNA, such as messenger RNA (mRNA), micro RNA (miRNA) and other small RNA species (Sork *et al*., 2018, Thakur *et al*., 2014, Lunavat *et al*., 2015). The nucleic acid cargo of EVs therefore holds high potential for both therapeutic and diagnostic applications, as gene expression in target cells could be manipulated with regulatory RNAs incorporated with the EVs, while EVs could also provide a snapshot to the genetic or transcriptomic profile of diseased cells. For instance, mesenchymal stem cell-derived EVs have been shown to possess regenerative properties in organ damage that are RNA-dependent (Cantaluppi *et al*., 2012, Zou *et al*., 2016). These EVs were able to induce a significant decrease of miR-125b and cell proliferation in recipient leukemia and breast cancer cells even *in vivo* in mice. Also non-modified mesenchymal stem cell-derived EVs have been shown to possess RNA-dependent organ regeneration-promoting properties (Zou *et al*., 2016). Compared to the cells of origin, cancerous or normal cells, the RNA profiles of EVs are enriched in smaller RNA species (25 - 1000 nucleotides), most of which are ribosomal or transfer RNA fragments and other more obscure RNA species with gene regulating properties, while the amount of miRNA and other regulatory RNA is lower (Wei *et al*., 2017, Hinger *et al*., 2018, Sork *et al*., 2018, Temoche-Diaz *et al*., 2019). Heterogeneity regarding the RNA size profiles has also been reported within EVs. EV-subtypes separated with differential centrifugation, density gradient or filtration approaches have been used to show that there are EV-size

dependent differences in their RNA-profiles, though the RNA enrichment patterns differ between individual studies (Wei *et al*., 2017, Lunavat *et al*., 2015, Temoche-Diaz *et al*., 2019). It is not yet understood, how DNA is included into EVs besides apoptotic bodies, but it has been shown to be present and taken up by the cells, affecting their gene expression (Waldenström *et al*., 2012, Cai *et al*., 2013, Cai *et al*., 2014, Kalluri, LeBleu, 2016, Takahashi *et al*., 2017). EVs have also been suggested to be a disposal mechanism for damaged, harmful DNA (Takahashi *et al*., 2017). Most of the DNA cargo has been suggested to being associated on the outer surface of EVs (Shelke *et al*., 2016, Németh *et al*., 2017, Lázaro-Ibáñez *et al*., 2019), which may hold true also regarding some of the RNA cargo. Therapeutic effect of EVs in renal injury has been reported being abolished by RNase treatment (Cantaluppi *et al*., 2012, Zou *et al*., 2016), while the miRNA cargo of EVs has been shown to be RNase resistant in other studies (Temoche-Diaz *et al*., 2019, Shurtleff *et al*., 2016), demonstrating that RNA localization may vary between EVs of different sources. Localization of nucleic acids on the outer surface indicates an endosomal pathway for their loading, since the plasma membrane pathway of EV biogenesis would entail the outer surface of forming EVs being in contact with the extracellular space, in contrast to the endosomal pathway of exosome biogenesis. RNA cargo in EVs appears to contain targeting zipcode sequences and hair pin structures that may guide them into EVs during cargo loading or provide structural stability during transport (Batagov, Kuznetsov & Kurochkin, 2011, Batagov, Kurochkin, 2013, Villarroya-Beltri *et al*., 2013, Bolukbasi *et al*., 2012). hnRNPA2B1 in cells has been identified as a key protein involved in miRNA loading into EVs, recognizing a GGAG motif that is enriched in miRNAs found in EVs (Villarroya-Beltri *et al*., 2013). Another zipcode was identified as a 25 nucleotide sequence with a CTGCC core that is recognized by miR-1289, which was found to mediate loading RNA into EVs (Bolukbasi *et al*., 2012). Several other miRNA-binding proteins have been identified as part of a sorting mechanism for different miRNA species to EVs (Temoche-Diaz *et al*., 2019, Shurtleff *et al*., 2016, Mukherjee *et al*., 2016, Lu *et al*., 2017), demonstrating how complex the process of RNA sorting into EVs, suggesting that it is a highly regulated process and therefore a significant part of cells' functions.

EVs have also been shown to contain metabolite profiles that differ significantly from their originating cells (Palviainen *et al*., 2019, Puhka *et al*., 2017). As metabolomics focuses on the analysis of the smallest molecules found in organisms, their chemical variety is much larger compared to other characterization methods. However, any conclusions regarding the biological

origin and relevance of the individual identified species are difficult to draw. EVs also possess enzymatic activities that can directly process and produce metabolites, therefore affecting the metabolome of recipient cells and even the one contained within EVs (Iraci *et al*., 2017). Nevertheless, certain enriched metabolic pathways have been found associated to the metabolites of EVs, including the urea cycle, amino acid, nucleotide and protein biosynthesis pathways (Palviainen *et al*., 2019, Puhka *et al*., 2017). As with other biomolecular cargo in EVs, their metabolome is affected by the state and type of the secreting cell (Palviainen *et al*., 2019). Therefore, the metabolic profile of EVs could be useful as a diagnostic tool or for monitoring the state of cell cultures.

1.1.3 Internalization of extracellular vesicles

In order to mediate their functions to cells, EVs can interact with them directly by signaling via receptors on the cell surface, such as MHC-I/-II to T-cells, leading to T-cell activation (Tkach *et al*., 2017), as presented in figure 3. Alternatively, EVs are internalized by the recipient cell, leading to their intraluminal cargo release into the cytoplasm. EVs have been shown to be able to use all of the cellular uptake mechanism for cell entry, including direct fusion with the plasma membrane, different forms of endocytosis, phagocytosis and macropinocytosis (Mulcahy, Pink & Carter, 2014, Murphy *et al*., 2019). Different cell types have been shown to prefer certain mechanisms. For example, phagocytes have been suggested to mainly phagocytose EVs (Feng *et al*., 2010), although also other pathways have been shown being used significantly (Popēna *et al*., 2018), while in most other cell types endocytosis appears to be the most common pathway (Tian *et al*., 2010, Tian *et al*., 2013, Svensson *et al*., 2013, Nanbo *et al*., 2013). Regardless of the pathway, internalization of EVs begins with an interaction between the vesicle and internalizing cell surfaces. Binding of the EVs to the cell's surface will eventually lead to the internalization of EVs via some of the many alternative pathways, though passive internalization of EVs has been excluded as EVs do not enter cells that have been fixed or kept at +4 °C (Tian *et al*., 2010, Tian *et al*., 2013, Svensson *et al*., 2013, Verdera *et al*., 2017). Receptor-mediated endocytosis and phagocytosis are triggered processes for the internalization of specific molecules, involving the formation of cargo-enveloping structures through transmembrane signaling induced by the cargo-bound receptor. However, the process of

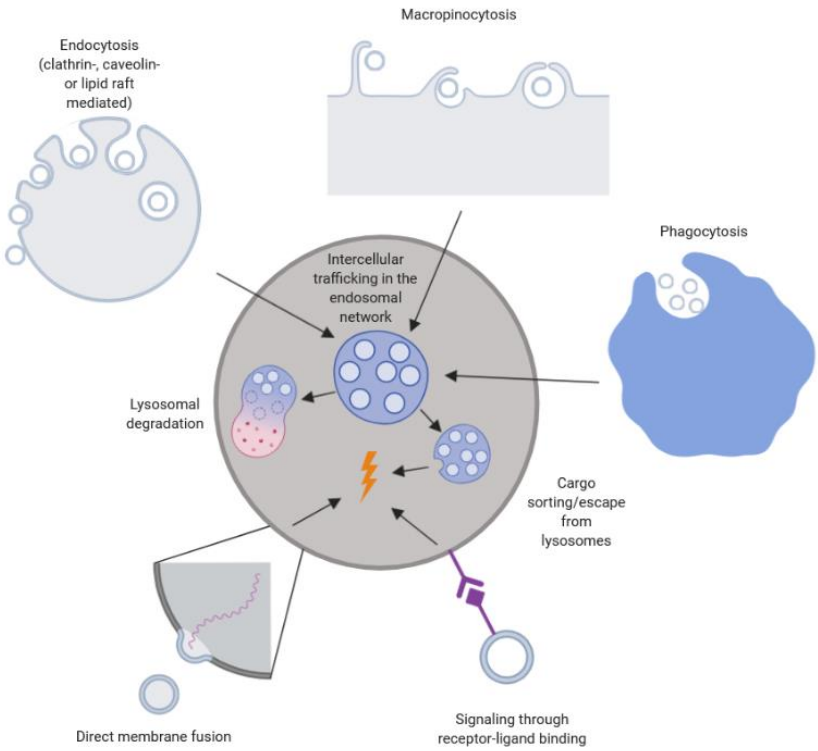


Figure 3: EV Internalization pathways and mechanisms for interactions with cells. EVs can interact with cells directly by binding to receptors on the cell membrane or after internalization by direct membrane fusion or different types of endocytosis, leading to a cellular response (lightning bolt). After internalization, whole EVs enter the endosomal network, which can lead to degradation by lysosomes or delivering their cargo to other cellular organelles.

passive membrane recycling occurs routinely in all cells, which would also lead to the non-specific internalization of any plasma membrane-bound EVs, although this process is slower than receptor-mediated endocytosis or phagocytosis. Macropinocytosis is also a non-specific process that cells use via cell membrane ruffles to uptake extracellular fluid, which can contain EVs as well in addition to any EVs bound to the plasma membrane, and therefore lead to their internalization. However, EVs and EV-associated endothelial growth factor have been shown to also promote macropinocytosis, and thus their own internalization through this pathway (Verdera *et al*., 2017, Nakase *et al*., 2015). Actin controlled cellular protrusions called filopodia have also been identified

as sites, where EV internalization appears to be enriched (Heusermann *et al*., 2016). Some commonly occurring surface structures relevant to EV-cell interactions have been identified that could lead to the non-specific internalization of EVs. These include glycan moieties such as heparin sulphate proteoglycans on both the cell and EV surfaces, lipid rafts (Svensson *et al*., 2013), and PS on EVs, which are recognized by several different receptors.

Following the endocytosis of intact EVs, they follow the endosomal network into their subcellular destination (Tian *et al*., 2010, Tian *et al*., 2013, Svensson *et al*., 2013, Nanbo *et al*., 2013, Ma *et al*., 2016). Internalized EVs have been observed to traffic to the endoplasmic reticulum (Heusermann *et al*., 2016), multivesicular bodies (Svensson *et al*., 2013) and lysosomes (Tian *et al*., 2013), though the exact fate of the cargo in EVs after internalization has not yet been clarified in detail. However, it has been shown that the lipids and proteins of EVs separate at some point inside the recipient cells, leading to EV protein accumulation to lysosomes, while the lipids may be retained as a part of the endosomal network and recycled to different membrane structures (Tian *et al*., 2010). Additionally, it was shown that inhibition of endosomal acidification prevented also the spreading of fluorescent lipid marker from EVs to the late endosome membrane (Nanbo *et al*., 2013). These observations suggest fusion of the EV membrane with the membranes of endosomes after internalization, leading to the release of the EV intraluminal cargo into the cytoplasm. In the case of fusion with the plasma membrane, the intraluminal cargo would be immediately released into the cytoplasm with the EV membrane integrating with the plasma membrane. The exact functions of these cargo molecules of course depend on their composition and structure, assuming that they survive degradation once inside the cell.

1.1.4 Functions of extracellular vesicles in cancer

While EVs participate in normally occurring functions that promote the physiological homeostasis of the host organism, cancer cells have been shown to secrete EVs that promote the progression of the disease. These EVs affect cells in their immediate vicinity and in more distal sites, both normal cells and other cancer cells. Several studies have reported that EVs from aggressive, i.e. quickly growing and spreading, cancer cells are more potent in promoting the different kinds of cancer promoting behavior in recipients than non-aggressive cancer cells. For instance, more metastatic cancer cells have been shown to promote a similar aggressive phenotype in other

cancer cells via EV-mediated delivery of oncogenes, causing increased invasiveness (Schillaci *et al*., 2017, Yokoi *et al*., 2017), drug resistance (Dorayappan *et al*., 2018) and proliferation (Ozawa *et al*., 2018), though EVs from non-aggressive cancer cells can also have similar effects (Raimondo *et al*., 2015, Bouvy *et al*., 2017). Manipulating the healthy cells of the tumor microenvironment with cancer cell EVs has also been demonstrated to promote the progression of the disease. For example, fibroblasts, endothelial cells and immune cells have been identified as important targets for these EVs, each with distinct roles in tumor development. EVs from Hodgins's lymphoma cells have been shown to attract and alter the phenotype of fibroblasts in the tumor microenvironment towards cancer associated fibroblasts, via the activation of inflammatory signaling pathways (Dörsam *et al*., 2018). Cancer associated fibroblasts secrete tumor growth supporting growth factors and RNA also within EVs (Richards *et al*., 2017, Nabet *et al*., 2017), demonstrating a bi-directional feedback loop that is at least partially mediated by EVs. As the tumor grows, it becomes hypoxic and requires improved blood flow to continue growing. For this purpose, EVs from cancer cells have been shown to promote angiogenesis in the immediate environment of the tumor by transferring different RNA species and proteins such as miR-23a (Hsu *et al*., 2017), VEGF (Feng *et al*., 2017) and Wnt4(Huang, Feng, 2017) to endothelial cells, inducing the formation of new blood vessels. Many studies have also provided evidence that cancer cell derived EVs contribute to the perceived immune evasion of tumors by affecting different types of immune cells. For example, prostate cancer cells have been found to secrete EVs that induce pro-inflammatory adenosine production in dendritic cells, inhibiting T-cell function (Salimu *et al*., 2017) and EVs from glioblastoma and colorectal cancer cells can modulate monocyte differentiation and affect their cytokine secretion to a generally more tumor benefiting profile (Popēna *et al*., 2018, de Vrij *et al*., 2015). Additionally, EVs from prostate and colorectal cancer cells have been found to inhibit T-cell proliferation and modulate a more tumor growth promoting, regulatory T-cell phenotype via TGF-β1-mediated signaling (Yamada *et al*., 2016).

As mentioned above, cancer cell derived EVs are also able to promote the formation of pre-metastatic niches, where future metastases will form. EVs secreted by metastatic cancer cells can migrate to future sites for metastases and alter the cells at their destination. As different types of cancer have been identified as having tropisms for metastasizing in specific tissues, it has been suggested that the EVs that they secrete share those tropisms, determined by the integrin signature of the EVs (Hoshino *et al*., 2015). Thus EVs of metastatic cancer cells could be crucial in

choosing the site of future metastasis by recognizing and modifying it before circulating cancer cells can attach. It has also been shown that EVs induce vascular leakiness (Hoshino *et al*., 2015, Schillaci *et al*., 2017, Hsu *et al*., 2017), which has been proposed as a possible precursor for the niche formation, allowing the EVs and cancer cells to diffuse into the parenchyma through the leaky blood vessels (Hoshino *et al*., 2015). At the niche site, EVs can induce pro-metastatic microenvironment formation as discussed above, including promotion of angiogenesis, recruitment of cancer associated fibroblasts and pro-metastatic neutrophils through epithelial TLR3 activation (Liu *et al*., 2016) and modifying the extracellular matrix with matrix metalloproteinases they carry (Yue *et al*., 2015). Regarding the migration of metastatic cancer cells themselves, in addition to promoting vascular leakiness, cancer cell-derived EVs have also been shown to be able to cause degradation of the blood-brain-barrier through miR-181c-mediated disruption of actin organization in brain endothelial cells, allowing metastasizing cancer cells to migrate through the barrier and into the brain (Tominaga *et al*., 2015). Cancer cells can also use EVs to promote a transition to a more mobile phenotype through endothelial-mesenchymal transition (Yue *et al*., 2015) or by inducing an amoeboid phenotype in the cells (Schillaci *et al*., 2017), which help the cancer cells' detachment from the tumor and their survival in circulation. Overall, cancer cell derived EVs target both other cancer cells and healthy cells, promoting tumor growth via many complex mechanisms that may be significant in the development of the disease. For this reason, it is believed that there is also a lot of potential in using EVs as biomarkers for diagnosing cancer, since EVs contain abnormal cancer-related biomolecules (Xu *et al*., 2018), and it has also been proposed that removal of cancer cell-derived EVs from the blood could be a powerful approach for cancer therapy (Nishida-Aoki *et al*., 2017).

1.2 Extracellular vesicles as carriers of therapeutic cargo for cancer treatment

In the last decade the scientific research in the field of EVs has grown exponentially, highlighting the rising importance and potential of EVs as carriers for targeted drug delivery, as reflected by the increase of publications (Figure 4). The key motivation towards developing EVs for therapeutic applications is that EVs are naturally occurring, functioning delivery vectors in humans and animals carrying biologically relevant information from cell to cell.

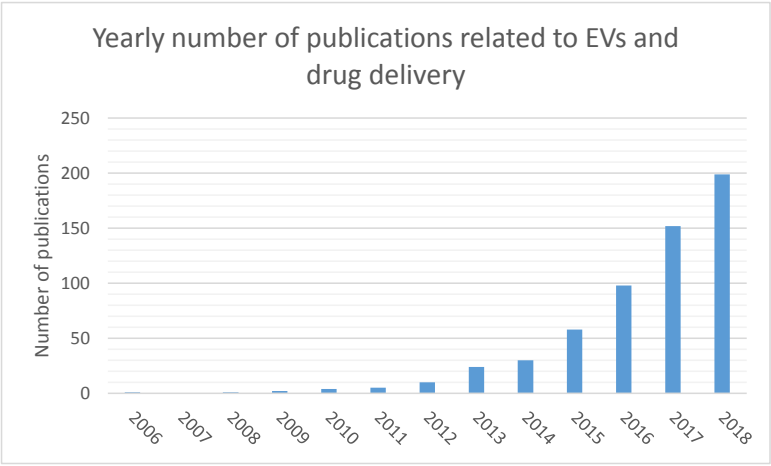


Figure 4: Bar graph showing the number of publications referring to the use of EVs in drug delivery. An advanced search was performed in the Web of Science (accessed 10.6.19) to find all articles from years 2006 - 2018 in English with the terms ("extracellular vesicles" OR ectosomes OR exosomes OR microvesicles") and "drug delivery". No articles matching the search criteria were found published prior to 2006.

Replacing or supplementing the natural cargo of EVs with a desired therapeutic load should therefore result in the delivery of therapeutics to the intended destination of the EVs. While EVs possess innate cell-mediated cargo loading and targeting properties, a major challenge for their use is posed by their rapid clearance by the mononuclear phagocyte system. In the following sections, these properties and challenges related to the preparation of therapeutic EVs are discussed in detail according to the current understanding of EV targeting and their engineering

for drug delivery purposes with a focus on cancer treatment. Examples of different cargo loading and targeting strategies with study outcomes are given at the end of section 1.2.3, in Table 1.

1.2.1 Targeting and clearance of extracellular vesicles

Targeted delivery of pharmaceuticals and biological therapeutics can enhance their desired effect dramatically while at the same time reducing the unwanted side-effects. In order to have any effect at all, therapeutics must reach the tissues, where they are needed. Because of their small size, it has been postulated that EVs would be well suited for cancer therapeutic applications due to the leaky vasculature of tumors, which leads to the passive accumulation of nanoparticles of approximately 100 nm in diameter, known as the enhanced permeation and retention (EPR) effect (Nichols, Bae, 2014). However, the EPR theory has been questioned based on the lack of success in human trials relying on EPR-mediated drug delivery (Nichols, Bae, 2014, Danhier, 2016) and the same negative result has been demonstrated in the case of EVs as well (Smyth *et al*., 2015). Therefore, the focus of therapeutic EV research is directed towards the development of actively targeting EVs carrying chemotherapeutics. Controlling the EV surface structures has been shown to translate to controlling their destination and interactions with the immune system. For example, it has been shown that the integrin signature of EVs is important in determining their target in metastatic organotropism, with the presence of integrins ITGα_6, ITGβ_4 and ITGβ_1 correlating with lung-tropism, while ITGβ_5 led to liver-tropism in EVs from different metastatic cancer cells (Hoshino *et al*., 2015). Also, other targeting proteins on EVs have been identified, such as tetraspanins that are enriched in EVs (Rana *et al*., 2012). In theory, any protein on EVs that recognizes a specific surface structure on the target cells could be used as a targeting moiety. Therefore, targeting of EVs could be accomplished by choosing appropriate cell lines expressing the desired targeting components (Sancho-Albero *et al*., 2019). Modifying the parent cells producing EVs has been performed in several studies to include targeting moieties to the EVs, with small peptides, membrane proteins and antibody fragments being the most prominent (Gudbergsson *et al*., 2019). Targeting peptides for example include a fragment of the rabies virus glycoprotein, which binds specifically to acetylcholine receptors, which are enriched in nervous and muscle cells (Alvarez-Erviti *et al*., 2011), the transmembrane domain of platelet-derived growth factor receptor targeting epidermal growth factor receptor on breast cancer cells (Ohno *et al*., 2013) and αv integrin specific peptide iRGD for breast cancer targeting (Tian *et al*., 2014).

Addition of targeting moieties to EVs via GPI-anchoring is another viable alternative, as it does not interfere with the functionality of existing proteins, as demonstrated by engineering of EVs with nanobodies targeting EGFR (Kooijmans *et al*., 2016a). Modifications have also been applied to EVs in post-isolation to include targeting moieties onto the EV surface, such as lipid-linked hyaluronic acid that binds to CD44 that is enriched in cancer cells (Liu *et al*., 2019) or peptides that bind EV-associated membrane proteins and can be used to crosslink cargo and targeting moieties to EVs (Gao *et al*., 2018). It is difficult to evaluate, whether there are any significant advantages gained from choosing either endogenous or exogenous targeting methods. As EVs from most cells are very heterogeneous in nature, any EVs not containing the targeting moieties should be removed from the preparation prior to use to prevent non-specific targeting if highest specificity is desired. In this sense exogenous targeting may have an advantage, as engineering EVs post isolation should affect the whole population equally, while endogenous targeting can be easier to perform once a stable cell line has been established. The validity of this conclusion still remains questionable as the universality of the methods mentioned above require further development and assessment by methods capable of high-throughput single EV analysis.

However, the clearance of non-modified EVs from blood is too high to achieve a reasonable exposure in targeted tissues (Kooijmans *et al*., 2016b). Improving the lifetime of EVs in circulation by reducing their nonspecific clearance is the most important aspect before even considering specific targeting of EVs. Especially macrophages contribute significantly to the rapid clearing of systematically administered EVs, leading to their accumulation in the mononuclear phagocyte system, especially in the liver and spleen (Imai *et al*., 2015). Elimination of macrophages (Imai *et al*., 2015) or inhibiting their scavenger receptor-mediated EV uptake with dextran sulfate (Watson *et al*., 2016) led to prolonged EV circulation time in mice and altered biodistribution patterns, with reduced accumulation in liver and spleen. Therefore, any therapeutic EVs should be modified to avoid recognition by macrophages, allowing them to reach their destination. Additionally, a strategy commonly used with liposome formulations involving poly (ethylene glycol) (PEG) coating (PEGylation) used to hide liposomes from phagocytes, has also been found applicable with EVs. PEGylation of EVs increased their circulation time, without affecting the biodistribution pattern (Kooijmans *et al*., 2016b). PEGylation of nanoparticles still has drawbacks that need to be taken into account in their formulations (Fang *et al*., 2017), as PEGylation hides the particles from other cells as well besides phagocytes, overcoming any targeting moieties they

may contain. An alternative strategy could be to overexpress the natural “don’t eat me” signals on the EVs, such as CD47, that are used by the immune cells to distinguish “self” from “not self” cells (Hayat *et al*., 2019). Indeed, it has been shown that EVs expressing CD47 are retained in circulation for a longer period than EVs from CD47-knockout cells (Kamerkar *et al*., 2017).

1.2.2 Loading therapeutic cargo into extracellular vesicles

Besides of the perspective on targeting, choosing the source of EVs for therapeutic applications is critical due to the differing innate content of EVs from different cell types that have differing effects on the cells once internalized. In contrast to targeting moieties that are found on the surface of EVs, therapeutic molecules can also be loaded inside of the EVs, making the selection of suitable EVs based on their cargo from a heterogeneous population of vesicles even more challenging. Cell lines enable production of EVs with desired biological cargo either through endogenous loading by transfection(Lang *et al*., 2017, Kim *et al*., 2018), overexpression of the cargo(Bolukbasi *et al*., 2012, de Jong *et al*., 2012, Mizrak *et al*., 2013), enrichment to EV biogenesis sites by guiding sequences in RNA(Batagov, Kuznetsov & Kurochkin, 2011, Batagov, Kurochkin, 2013, Villarroya-Beltri *et al*., 2013, Bolukbasi *et al*., 2012), ubiquitination(Liu *et al*., 2009), linking them to lipid anchors(Kooijmans *et al*., 2016a) or fusion with EV-enriched proteins (Tian *et al*., 2014, Sutaria *et al*., 2017). Besides endogenous cargo sorting, exogenous loading of biological molecules into EVs is challenging, given their large molecular size and ionic charge, which makes it difficult to transport them across a nonpolar lipid membrane surrounding the EVs. Experimental data regarding post-isolation loading of biomolecules has been focused mainly on small RNA species such as miRNA and small interfering RNA (siRNA) due to their functions in post-transcriptional gene silencing and relatively small size, making them potent candidates for therapeutic cargo. Post-isolation loading of EVs requires generating temporary holes in the EV membrane by electroporation, sonication, extrusion, hypotonic dialysis or saponin treatment (Fuhrmann *et al*., 2015, Lamichhane, Raiker & Jay, 2015, Lamichhane *et al*., 2016, Kim *et al*., 2016, JC Bose *et al*., 2018). Similar strategy can be applied for hydrophilic small molecule drug loading. This approach is inefficient though, since there is no active driving force for the cargo to enter the EVs. In effect, it relies on random diffusion through small, temporary holes into small particles that, depending on their concentration, often occupy only a minute portion of the sample volume. Additionally, electroporation has been shown to cause aggregation of siRNA, which may hinder

loading efficiency if the method is not optimized properly (Kooijmans *et al*., 2013). Despite these issues, electroporation has been used in several studies for the preparation of EVs carrying various cargos, including siRNA, with promising therapeutic outcomes (Alvarez-Erviti *et al*., 2011, Kamerkar *et al*., 2017, Usman *et al*., 2018). Loading of hydrophobic molecules on the other hand can be accomplished easier, since they have high binding affinity to the nonpolar lipid membrane of the EVs, which also increases their solubility when compared to a simple water solution (Fuhrmann *et al*., 2015, Kim *et al*., 2016, Sun *et al*., 2010). Hydrophobic molecules can therefore be loaded simply by incubating them with EVs, although the process can be further enhanced by increasing the fluidity of the EV membrane, for instance by sonication (Kim *et al*., 2016). Nucleic acids such as siRNA can also be loaded onto EVs by incubation when they are crosslinked to lipophilic molecules such as cholesterol (Didiot *et al*., 2016, O'Loughlin *et al*., 2017). Small molecule drugs and nanoparticles can also be loaded into EVs by feeding them to the producing cells, from which they are carried over to the EVs they secrete (Sancho-Albero *et al*., 2019, Liu *et al*., 2019, Tang *et al*., 2012). Promising alternative methods have also been reported for loading EVs, for example by fusing them with liposomes, creating EV-liposome hybrid vesicles (Sato *et al*., 2016, Piffoux *et al*., 2018). The liposome fusion method seems promising, given that liposomes can be prepared with precise lipid composition and content, including RNA, which could then be transferred to the EVs via fusion.

1.2.3 Delivery of cancer therapeutics with extracellular vesicles

The anti-cancer effect of EVs loaded with different chemotherapeutic drugs has been assessed in both *in vitro* and *in vivo* studies with promising results. EVs containing doxorubicin or the anti-inflammatory compound curcumin could even cross the blood-brain-barrier carrying the drugs with them in mice with an intranasal delivery route (Zhuang *et al*., 2011) or in zebrafish with an intravenous injection (Yang *et al*., 2015). This resulted in reduced inflammation in the case of curcumin in a brain tumor model, leading to reduced tumor growth (Zhuang *et al*., 2011). Doxorubicin-carrying EVs also reduced the growth of brain cancer cells significantly (Yang *et al*., 2015). Other drugs used with EVs include paclitaxel (Kim *et al*., 2016, Tang *et al*., 2012, Yang *et al*., 2015) and cisplatin (Tang *et al*., 2012) that are commonly used in chemotherapy and reported to have increased cytotoxicity in cancer cells, when delivered with EVs targeted to these cells. Besides increasing the cytotoxicity of the drugs towards cancer cells, the EV-mediated delivery was

also found to reduce the harmful side-effects of the drugs. For example, the inherent cardiotoxicity hinders the use of doxorubicin in cancer therapy (Singal, Iliskovic, 1998), which is why a commercial formulation of doxorubicin packed in PEGylated liposomes (Doxil) is available. However, with EV-mediated targeted delivery to αv integrin expressing cancer cells, the concentration of doxorubicin in the heart could be limited as well without PEGylation (Tian *et al*., 2014). Additionally, it was shown that the presence of gap junction protein connexin 43 in EVs with doxorubicin could further reduce its cardiotoxic effect without compromising its chemotherapeutic effect against cancer cells (Martins-Marques *et al*., 2016). EVs carrying doxorubicin and PTX could also overcome multidrug resistance in cancer cells, normally making these cells unaffected by the drug (Kim *et al*., 2016). With EVs, these drugs were able to bypass the P-glycoprotein-mediated drug efflux from the cells because of the endosomal delivery route, increasing the cytotoxicity of the drugs towards the resistant cells significantly. It has also been suggested that some EVs can be used to increase drug sensitivity of cancer cells without additional cargo or endogenous modifications (Ma *et al*., 2016, Liu *et al*., 2019, Jin *et al*., 2017). It was found that EVs secreted by tumor cells after UV irradiation can inhibit P-glycoprotein expression (Ma *et al*., 2016) and promote lysosome mediated trafficking of the chemotherapeutics to the nucleus, thus increasing the cytotoxicity of DNA binding drugs (Ma *et al*., 2016, Liu *et al*., 2019).

Besides small molecule chemotherapeutics, EVs have also been used to deliver biomolecular cargo that affect cancer cells more specifically. Several miRNA and siRNA species have been identified with cancer suppressing activities, but their delivery requires a carrier that protects them from extracellular nucleases and helps them cross the cell membrane, a role that EVs can fulfill. Studies with such RNA species including miR-134 (O'Brien *et al*., 2015), let7 (Ohno *et al*., 2013) and siRNA (Alvarez-Erviti *et al*., 2011) have shown proof of concept for the effective anti-cancer delivery of regulatory RNAs that show little to no adverse side effects. Therapeutic mRNA and protein cargoes have also been utilized, for example to sensitize cancer cells to specific drugs, such as cytosine deaminase that converts prodrug 5-fluorocytosine into cytotoxic 5-fluorouracil (Mizrak *et al*., 2013), providing another treatment option with little side effects. Another interesting strategy of EV-mediated therapy is to deliver oncolytic viruses that are able to specifically infect cancer cells, however the use of oncolytic viruses is limited by the strong immune response that they generate, leading to their elimination from blood circulation and thus preventing them from reaching cancer cells (Lawler *et al*., 2017). Additionally, viruses often

depend on specific receptors on the cell surface for binding and entering the cells, which may be absent from cancer cells. EVs could help circumvent these issues by providing both camouflage against the immune cells and complement proteins, while providing alternative modes of entry into the cancer cells. Although numerous studies have shown that viruses may use EVs to carry infective or non-infective viral cargo as reviewed by (Raab-Traub, Dittmer, 2017, van der Grein, Susanne G *et al*., 2018), only a few studies have focused on their therapeutic potential. A study by Ran *et al*. (Ran *et al*., 2016) showed that apoptotic cancer cells infected with an oncolytic adenovirus produce large infective EVs. These EV-encapsulated virions were resistant towards adenovirus neutralizing antibodies and were able to infect other cancer cells, even those lacking the CAR protein that adenovirus in question (isotype 5) requires for cell binding and internalization. Adeno-associated virus (AAV)-carrying EVs have also been explored as potential mediators for gene therapy, with the EV membrane protecting the virus from antibodies while achieving effective transduction in target cells and allowing retargeting of the virus by manipulating the EV surface (Maguire *et al*., 2012, György *et al*., 2017, Wassmer *et al*., 2017).

Table 1: Assessment of studies evaluating EVs for cancer therapy. Example studies regarding different therapeutic EV preparation methods and their assessment are presented, with the respective preparation method, added targeting and cargo molecules and key outcomes listed.

Reference	**Method for EV loading**	**Active targeting**	**Therapeutic cargo**	**Outcomes**
(Alvarez-Erviti *et al*., 2011)	siRNA loading by electroporation into dendritic cell EVs	Fusion of Lamp2b with peptides (RVG and MSP) targeting neural and muscle cells	siRNA against BACE1, a gene involved in Alzheimer's disease	Significant *in vivo* BACE1 inhibition and amyloid plaque formation in the brain of mice after intravenous administration of BACE1 siRNA carrying RVG-targeted EVs.
(Liu *et al*., 2019)	UV irradiation of donor cells followed by incubation with doxorubicin	Lipid conjugated hyaluronic acid	Doxorubicin	Hyaluronic acid mediated targeting of doxorubicin-carrying EVs towards CD44 expressing multidrug

				resistant breast cancer cells. The EVs increased the cytotoxic effect of doxorubicin in cancer cells, its blood circulation time and reduced its cardiotoxic side effects.
(Fuhrmann *et al*., 2015)	Porphyrin loading by passive incubation, sonication, electroporation, saponin treatment and hypotonic dialysis into EVs from several cell lines	None	Porphyrins of different water solubilities	Hydrophobic porphyrins were loaded more effectively than hydrophilic. EVs increased their cellular uptake and toxic effect. Saponin and hypotonic dialysis treatments resulted in the highest loading efficiencies.
(Piffoux *et al*., 2018)	PEG-mediated EV-liposome fusion with EVs from HUVEC cells	PEGylation to reduce clearance by phagocytosis	Photosensitizer mTHPC	Loading efficiency of mTHPC was close to 90 % with liposome fusion, preserving the natural cargo of EVs as well. Transfer of PEG to EVs also reduced their interactions with macrophages. Compared to commercial liposomes, EVs delivered more mTHPC to cancer cells.
(Yang *et al*., 2015)	Incubation of bEND.3 EVs with paclitaxel or doxorubicin at +37 °C	None	Paclitaxel and doxorubicin	Successful doxorubicin delivery across the blood-brain-barrier in zebrafish embryos, with reduced tumor size.

(Ran *et al*., 2016)	EVs from A549 cancer cells infected with oncolytic adenovirus	None	Oncolytic adenovirus	Improved tumor reduction in mice and protection from adenovirus neutralizing antibody.
(Gao *et al*., 2018)	C2C12 myoblast-derived EVs incubated with a CD63-binding peptide (CP05), linked with cargo and targeting moieties	Various peptides targeting different tissues. For therapeutic assessment, M12 peptide targeting to muscles was used	Phosphorodiamidate morpholino oligomer (PMO), an antisense oligonucleotide for the treatment of Duchenne's muscular dystrophy	CP05 enabled CD63-mediated binding of EVs from different sources and attaching multiple moieties. Intravenous administration of EVs carrying both CP05-M12 and -PMO lead to effective delivery of PMO to muscle cells, improving dystropin expression

In conclusion, EVs provide a natural means to deliver various therapeutic cargo that can be adapted for the treatment of cancer. EVs can be loaded both with biological and synthetic molecules that possess targeting and therapeutic properties, enabling safer and more precise treatment of cancer in the future. However, the development of EVs for any therapeutic applications requires better understanding of the mechanism of EV-mediated cargo delivery and further development of methods for the production of therapeutic EVs.

2 Aims of the thesis

The objective of this thesis was to produce EVs carrying chemotherapeutic cargo and to examine their cytotoxic effect and mechanisms of cargo delivery into cancer cells *in vitro*. Autologous cancer cell-derived EVs were used as the model EVs for cargo delivery, with paclitaxel and an oncolytic adenovirus as the model therapeutics. More specific aims were as follows:

Production of paclitaxel-carrying EVs by passive loading after EV isolation and determination of the cytotoxicity of the loaded EVs. (paper I)

Examination of the EV-mediated mechanism of paclitaxel delivery by assessing its dependence on dose and surface proteins and by visualization by live cell microscopy. (papers I & II)

Production and characterization of oncolytic adenovirus-carrying EVs by infecting cancer cells, followed by EV isolation from infected cell cultures. (paper III)

3 Materials and methods

3.1 Cell culture (I, II & III)

Prostate cancer cell lines LNCaP and PC-3 and lung cancer cell line A549 were obtained from the American Type Culture Collection. All cell types were grown in T-175 flasks to 80 % confluence (I & III) or continuously in a two-chamber bioreactor (II) at +37 °C and 5 % of CO_2. LNCaP cells were grown in Roswell Park Memorial Institute 1640 medium (RPMI), PC-3 and A549 cells in Dulbecco modified Eagle medium (F12k), both supplemented with 5 or 10 % (v/v) fetal bovine serum (FBS) and penicillin/streptomycin (100 units/mL penicillin and 100 µg/mL streptomycin). FBS was

vesicle-depleted by an overnight ultracentrifugation at 110 000 × *g*, followed by filtration with 0.22 µm Steritop filter (Millipore) (I & III).

II: The bioreactor upper compartment was filled with 750 mL of Ham's F-12 K medium for PC-3 cells with non-depleted 10 % FBS, supplemented to a final concentration of 4.5 g/l glucose solution, and 100 units/mL penicillin/streptomycin. 15 mL of corresponding Ham's culture medium without FBS was used in the bioreactor's cell compartment to avoid FBS derived EVs.

III: For infecting the cells, the medium with 5 % FBS was also supplemented with Ad5/3-D24-GMCSF oncolytic adenoviruses at a concentration of approximately 30 particles per cell.

3.2 EV isolation

3.2.1 Differential centrifugation (I, II & III)

EVs were isolated from conditioned medium using differential ultracentrifugation.

I & II: First centrifugation at 2500 × *g* for 25 min at +4 °C to remove cell debris, followed by 20 000 × *g* for 60 min at +4 °C to obtain the 20K MVs. The final supernatant was ultracentrifuged at 110 000 × *g* for 1 h at +4 °C to obtain the 110K EXOs using rotor type 50.2Ti (Beckman Coulter).

III: First centrifugation at 500 x g for 10 min at +4 °C to pellet cells, followed by 15 000 x g for 15 min at +4 °C to pellet cell debris and ultracentrifugation at 150 000 x g for 2 h at +4 °C using rotor SW32Ti (Beckman Coulter) with 200 µL of 45% (w/v) iodixanol added to the bottom of the tubes as a density cushion. The bottom 3 mL of each tube was then pooled and concentrated further to 250 µL with an Amicon Ultra-15 10 kDa centrifugal ultrafiltration unit (Millipore) at +4 °C.

3.2.2 Iodixanol density gradient centrifugation (II & III)

II: EV-pellets were loaded on top of a gradient prepared by overlaying 700 µl of 10 % iodixanol on top of 300 µl of 30 % iodixanol in 1.5 mL ultracentrifuge tubes. The samples were then ultracentrifuged in rotor TLA-55 with 170 000 x g for 3 h at +4 °C. The resulting visible EV-band in the 10 % and 30 % interphase was then collected and iodixanol was removed by ultrafiltration, filtering the samples three times with Amicon Ultra-4 10 kDa ultrafiltration units.

III: The density gradient was prepared with the diffusion method in a 13.5 mL ultracentrifuge tube by overlaying 4 mL of 45 % iodixanol with 4 mL of 25 % iodixanol buffered with Dulbecco's phosphate buffered saline (DPBS)-2 mM $MgCl_2$ and filling the rest of the tube with the same buffer. The tube was then incubated for one hour in horizontal position at +22°C. The samples mixed with iodixanol were then loaded through the gradient to the bottom of the tube and ultracentrifuged at 200 000 x g for 3 h at +4 °C with rotor SW41Ti (Beckman Coulter). After the run 1 mL fractions were collected from the top of the tubes for further analyses, measuring their densities gravimetrically.

3.2.3 Isolation of oncolytic adenoviruses (III)

Ad5/3-D24-GMCSF virions were isolated from dead, infected A549 and PC-3 cells lysed by four freeze-thaw cycles and the cellular debris was removed by centrifugation with 5000 x g for 10 min at +4 °C. An iodixanol gradient was prepared into a 13.5 mL ultracentrifuge tube by layering 2 mL of 40 %, 3 mL of 25 % and 4 mL of 15 % iodixanol diluted in DPBS containing 2 mM $MgCl_2$, with the 15 % solution containing 1 M NaCl. Rest of the tube was filled with the virus-containing supernatant and ultracentrifuged at 200 000 x g for 2 h at +4 °C with rotor SW41Ti. The resulting band of viruses just below the 40 % and 25 % iodixanol interphase was collected and iodixanol was removed by ultrafiltrating the samples three times with DPBS, using Amicon Ultra-4 10 kDa ultrafiltration units at +4 °C.

3.3 Immunoblot analysis of proteins (I, II & III)

For western blotting, the protein markers present in cells and EVs were analyzed by loading equal protein amounts determined by a bicinchoninic acid (BCA) protein assay kit (Thermo Scientific). Whole cell lysates and EVs were prepared in 2 × Laemmli buffer (Bio-Rad) under non-reducing or reducing conditions depending on the antibodies used, separated with SDS-PAGE and the proteins were transferred onto a nitrocellulose membrane. For dot blot analysis, equal volumes of sample were blotted directly onto the membrane without prior separation. The membrane was then blocked with 5% (w/v) skim milk powder or BSA in Tris-buffered saline-0,1 % (v/v) Tween 20 (TBS-

T) and then incubated with the respective primary antibody and secondary horseradish peroxidase (HRP)-linked antibody and imaged using either X-ray film or chemiluminescence imaging system.

3.4 Nanoparticle tracking analysis (NTA) (I, II & III)

Purified EV samples were analyzed by NTA using Nanosight model LM14 (Nanosight) equipped with blue (404 nm, 70 mW) laser and SCMOS camera. One 60 s video (III) or three 90 s videos (I & II) were recorded per sample and analyzed using NTA software 2.3 (I) or 3.0 (II & III). Viral samples were heat-inactivated at +95°C for 15 min prior to measurement.

3.5 Preparation of Paclitaxel-loaded EVs (I)

A 50 mM stock solution of Paclitaxel (PTX; Selleck Chemicals) was prepared by dissolving PTX into DMSO-isobutanol (1:1; Sigma-Aldrich). PTX-loaded EVs were prepared by incubating 1×10^8–5×10^9 EVs/mL in 1 mL of 5 µM PTX-DPBS solution for 1 h at +22 °C. For trypsin-treated PTX-EVs, 0,25 % trypsin was added to the samples and incubated for 30 min at +37 °C. Next, the samples were centrifuged at 170 000 × *g* for 2 h at +4 °C to pellet the EVs. The supernatant containing unbound PTX and trypsin was removed, and the EV-pellet was washed by suspending it in DPBS and pelleting it again.

3.6 Fluorescent labeling of EVs (I & II)

I: EVs were double-labeled with DiD lipophilic dye (Biotium) and OregonGreen-labeled PTX (OG-PTX), (Invitrogen) to study the cellular distribution of PTX-EVs. First, the EVs were incubated for 1 h at +22°C with 5 µL of DiD solution per mL of EV suspension in DPBS. The unbound dye was removed by size exclusion chromatography (SEC) and the particle concentration was determined by NTA before labeling with OG-PTX. Next, 5×10^9 vesicles were incubated with 1 µL of OG-PTX (5 µM final concentration) in 1 mL of DPBS for 1 h at +22°C and purified by ultracentrifugation. For the co-localization studies of EVs and endosomes/lysosomes, the EVs were labeled with DiO lipophilic dye (Biotium). For the uptake experiments, EVs were labeled with 2 µg/mL of DilC18(5)-

DS red dye (Life Technologies) for 20 min at +37 °C. Unbound dye was removed by pelleting the EVs twice by ultracentrifugation at 170 000 x g for 2 h at +4 °C.

II: The 20 K MVs and 110 K EXOs were labeled and loaded for fluorescence lifetime imaging microscopy (FLIM) imaging by adding 7.5 µl of 1 mM PTX-OG to one milliliter of 20K MV/110K EXO suspension (20 K MV/110 K EXO particle concentration adjusted with DPBS to 1.5×10^{10} particles/mL), and by incubating the suspension protected from light for 60 min upon shaking at ambient temperature. Following the incubation, the excess of PTX-OG was removed by centrifuging once with Optima MAX ultracentrifuge (MLA-130 rotor) at 110 000 g for 180 min, +22 °C. The 20 K MV/110 K EXO pellets were resuspended in 50 µl of DPBS before the application to the cells. After the PTX-OG labeling and loading, the MV membranes were labeled with DiD for confocal imaging by adding 1 µl of DiD to 1 mL of MV suspension (10^{10} particles/mL) and incubating for 30 min at +22 °C protected from light. Unbound DiD label was removed by centrifugation as described above.

3.7 Viability assays (I & III)

5000 cells per well were seeded in a 96-well plate and grown for two days before test sample administration. Cell viability was measured after 24 to 72 h by using the AlamarBlue viability assay according to manufacturer's instructions (Life Technologies). Fluorescence intensity was measured using VarioSkan Flash multireader (ThermoScientific) with 560 nm excitation and 590 nm emission filter settings. The viability values were then calculated from the fluorescence intensity values as a percentage of the control cells after subtracting the mean background values.

3.8 Extracellular vesicle uptake assessment by flow cytometry (I)

5×10^5 PC-3 cells and 3×10^5 LNCaP cells were seeded in 6-well plates at 70% confluence. The cells were then washed with DPBS and incubated with cell culture medium containing 10^8–10^9 particles/mL of DiD-labeled 20K MVs or 110K EXOs. The cells were incubated with their autologous EVs for 1, 3, 6, 9, 12, 24, 32, and 48 h. Cells without EVs were used as negative controls. At each time point, the supernatant was removed and the wells were washed two times with DPBS. Cells were detached by trypsin treatment and fixed with 4% paraformaldehyde in DPBS

for 15 min at +37 °C. 10 000 events were analyzed by flow cytometry (Gallios, Beckman Coulter) using 638 nm laser for excitation, measuring the geometrical mean fluorescence intensity of the cells, representing cell-associated EVs. Data was processed by using FlowJo software 10.0.

3.9 Fluorescence microscopy (I, II & III)

3.9.1 Fluorescence microscopy of infected cells (III)

For the apoptosis assay with Hoechst 33342 and propidium iodide (PI) staining, the cells were seeded in the wells of a 12 well plate and infected as described above. Every 24 hours following the initial infection the cells were stained with 100 µg/mL of both Hoechst 33342 (ThermoFisher Scientific) and PI (ThermoFisher Scientific), centrifuged with 1000 g for 5 minutes and inspected with EVOS FL Imaging System.

For the immunofluorescence staining of adenovirus hexon coat protein, the cells were washed at every 24 hours following the initial infection with DPBS and fixed with 4 % (w/v) paraformaldehyde for 10 minutes at room temperature. The cells were washed with DPBS, blocked with 5 % BSA-TBS-T, permeabilized with 0.1 % (v/v) Triton X-100 and stained with anti-Ad hexon antibody (8C4, Abcam) (1:2000 in 5 % BSA-TBS-T) and Alexa Fluor 488 goat anti-mouse secondary antibody (ThermoFischer Scientific) (1:200 in 5 % BSA-TBS-T) and inspected with EVOS FL Imaging System.

3.9.2 Live cell confocal microscopy (I & II)

Microscopy studies were performed using a TCS SP5II HCS A (Leica) confocal microscope and a 3I Marianas (3I intelligent Imaging Innovations) wide field microscope. On the imaging day, the cells were given the labeled EVs, free PTX, PTX-EVs or their combination. The cells were followed for the first 6 h, recording z-stacks covering the cells' thickness, and then additional images were taken after 24 and 48 h of incubation. The cell membranes were labeled with CellMask Deep Red Plasma membrane label and the lysosomes and endosomes with Lysotracker Red (Invitrogen, California, USA) prior to the addition of labeled EVs.

3.9.3 Fluorescence lifetime imaging microscopy (FLIM) (II)

Fluorescence lifetime images were acquired using a fluorescence lifetime microscope MicroTime-200 (PicoQuant, Germany) coupled to the inverted microscope Olympus IX-71 (Olympus, Japan). The pulsed laser diode LDH-P-C483 (PicoQuant, Germany) emitting at 483 nm (time resolution 120 ps) was used for fluorescence excitation and the emission was monitored using 510 nm long pass filter. The SymPhoTime 64 software was used to calculate the lifetime map images. To gain more information on the lifetime changes, a region of interest (ROI) was selected and the corresponding fluorescence decay was extracted.

3.10 qPCR analysis of viral DNA (III)

DNA extraction and analysis were performed by extraction of the genomic DNA (gDNA) using NucleoSpin Tissue kit (Macharey-Nagel, Düren, Germany) according to the manufacturer protocol. qPCR was performed with equal volumes of gDNA samples using Maxima Probe/ROX qPCR Master Mix (ThermoFisher Scientific) and LightCycler480 system (Roche, Basel, Switzerland) with following cycling conditions: +95 °C for 10 min, 50 cycles of (+95 °C for 15 s, +60 °C for 1 min).

DNA concentrations (in arbitrary units) for comparative analyses were then calculated from the acquired Ct-values with the formula

$[DNA] = 2^{-Ct}$,

and used to compare paired samples.

3.10.1 Proteinase K and DNase treatments

In order to examine the whether the DNA was inside IEVs, the samples were treated with proteinase K to free any DNA inside virus capsid, followed by degradation with DNase I. A sample of purified free virus was used as a control for the treatment. Samples were treated with 1 mg/mL proteinase K (ThermoFisher Scientific) for 2h in +37 °C. Proteinase K was inactivated by heating (+90 °C for 5min) followed by addition of complete protease inhibitor cocktail (Roche). 5 U of DNase I (ThermoFisher Scientific) was then added and samples incubated for 1h in +37 °C.

Treatment was ended by addition of 1:10 volume of EDTA and incubation of 10 min in +65 °C. The samples were then used for gDNA extraction as described above.

3.11 Transmission electron microscopy (TEM) (I & III)

3.11.1 TEM of EVs (I)

4 µL of EV samples were added onto purified EM grids, washed with distilled water, negatively stained with 2% aqueous uranyl acetate (System Biosciences, Mountain View) for 2 min and dried out protected from light for 20 min. The samples were analyzed by TEM (FEI Tecnai 12).

3.11.2 TEM of cells treated with infected cell-derived extracellular vesicles (III)

200 000 cells were seeded on top of EM grade 0.1 mm coverslips in and treated with IEV samples. After one or two days of incubation the cells were fixed with 2 % (v/v) glutaraldehyde for 20 minutes at +22°C. The cells were washed two times with 0.1 M Na-phosphate buffer pH 7.0 and osmicated with 1 % OsO_4 in 0.1 M NaCac buffer for 1h at RT. The samples were washed and dehydrated with ethanol, dipped in acetone and covered with Epon for 2h at RT and 18h at +60°C. 60 nm sections were cut from the samples onto pioloform grids and post-stained with 0.5 % uranyl acetate for 30min and 3 % lead acetate for 1min. Imaging was performed with Jeol JEM 1400 electron microscope (Akishima, Tokyo, Japan) operated at 80 kV.

3.11.3 Cryo-TEM of EV-/IEV-samples (I & III)

Iodixanol or sucrose was removed by ultrafiltration with Amicon Ultra-0.5 10 kDa ultrafiltration units from fresh, non-frozen samples (III). The vitrified samples were prepared with a Leica EM GP vitrification device from 3 µL aliquots of each sample on freshly prepared sample grids and observed in a JEOL JEM-3200FSC field emission Cryo-TEM (I) or FEI Talos Arctica microscope (III). The images were recorded at a magnification of 57,000× with a FEI Falcon 3 camera operated in linear mode.

4 Results

4.1 The effect of paclitaxel loaded extracellular vesicles on cancer cell viability was dose-dependent

EVs were isolated from two prostate cancer cell lines (PC-3 and LNCaP) with differential centrifugation into two populations based on their sedimentation speeds (20K MVs and 110K EXOs) and loaded with PTX by incubating at room temperature. Because PTX is a strongly hydrophobic compound, it was hypothesized that it would passively bind to the lipid membrane of EVs. Since there was much uncertainty in the results derived with LNCaP cells due to the cells' sensitivity to the cell culture procedures during viability assays, the results and discussion is focused on PC-3 cells, while the results obtained with LNCaP cells followed a similar trend. According to UPLC measurements of PTX concentration, approximately 10 % of the PTX in solution was bound to the EVs in the final preparation with no significant differences caused by the type or concentration of EVs during loading. The PTX-EVs were then given to their originating cells, and their cytotoxicity was assessed in a dose-dependent manner after 24 and 48 hours of incubation and compared to free PTX corresponding to the loading conditions (Figures 5A and B). While the presence of residual free PTX in the samples cannot be excluded due to leaking from EVs or as a result of aggregation during washing away of free drug, the resulting cytotoxicity profiles revealed several differing characteristics of PTX-EVs compared to free PTX. Firstly, the highest concentrations of PTX-MVs had reduced cell viability after 48 hours compared to the free PTX control (Figure 5B). In other words, MVs increased the cytotoxic effect of PTX. This effect was not apparent at the 24-hour time point, where the cell viability was higher in the PTX-MV groups compared to the free PTX control, suggesting an alternate, delayed mechanism of action of MV-mediated PTX delivery. In contrast, PTX-EXOs did not increase the cytotoxicity of PTX, remaining slightly less effective than the free PTX control. The lowest concentrations of PTX-EXOs actually increased cell viability, which peaked at the 48-hour time point. A much lesser viability enhancing effect was also observed with the lowest concentrations of PTX-MVs as well. This became apparent only when the concentration of PTX was low enough. Finally, the dose dependences of both PTX-EV types followed a similar s- curve type in cytotoxicity, with a saturation point around

the 10^8 EVs/mL concentration, where cytotoxicity was most strongly influenced by changes in concentration.

Next, to maximize the cytotoxicity of PTX-EVs, the amount of EVs was reduced during the loading of PTX to the saturation point concentration of 10^8/mL observed in viability assays. This lead to the increase of PTX per vesicle by a factor of 50 in the final preparation, designated as "high load-PTX-EVs" (HL-PTX-EVs) in contrast to "low load-PTX-EVs" (LL-PTX-EVs) in figure 5, after washing and improved the cytotoxicity of PTX-EXOs, while the cytotoxicity of PTX-MVs was unaffected (Figures 5C and D). HL-PTX-EXOs with more PTX per vesicle than in LL-PTX-EXOs were as effective as HL-PTX-MVs, also with reduced viability enhancement in the lower dose group compared to the EXOs with less PTX in Figure 5A. The viability enhancing effect of EVs was confirmed with MVs and EXOs without PTX (Figures 5E and F), where a similar pattern of viability increase was observed in both groups, confirming that it is a property of EVs related to the EV subtypes. Additionally, the dependence of the EV-mediated PTX delivery on EV surface proteins was assessed by removal of those proteins by trypsin treatment. The effect on the cytotoxicity of HL-PTX-EVs caused by the trypsin treatment (Figure 6) suggested that EV-mediated PTX delivery was mostly independent on the EV surface proteins, since their removal did not affect the cytotoxicity of HL-PTX-EXOs, although HL-PTX-MVs were slightly less cytotoxic at lower concentrations (Figures 6A and B). A similar effect was found, when comparing the uptake of labelled EVs by flow cytometry (Figures 6C and D). Non-treated EVs from both cell lines displayed similar accumulation patterns over 24-hours (Figure 6C), but trypsinization lowered the amount of MV uptake, while EXOs were unaffected (Figure 6D). Cryo-TEM analysis of 20K MVs did not show any structural changes caused to the vesicles by the treatment (Figures 6E and F), while western blot analysis showed substantially diminished signal from EV membrane proteins CD9 and CD63 (Figure 6G), suggesting that the observed difference in cytotoxicities was due to the cleavage of proteins, and not structural damage to the EVs.

Overall, these results show that autologous EVs are able to bind PTX in solution and carry it into prostate cancer cells. This delivery mode can enhance the cytotoxicity of PTX but the natural cargo of EVs can overcome the drug's effect. The formulation of PTX per vesicle appears to

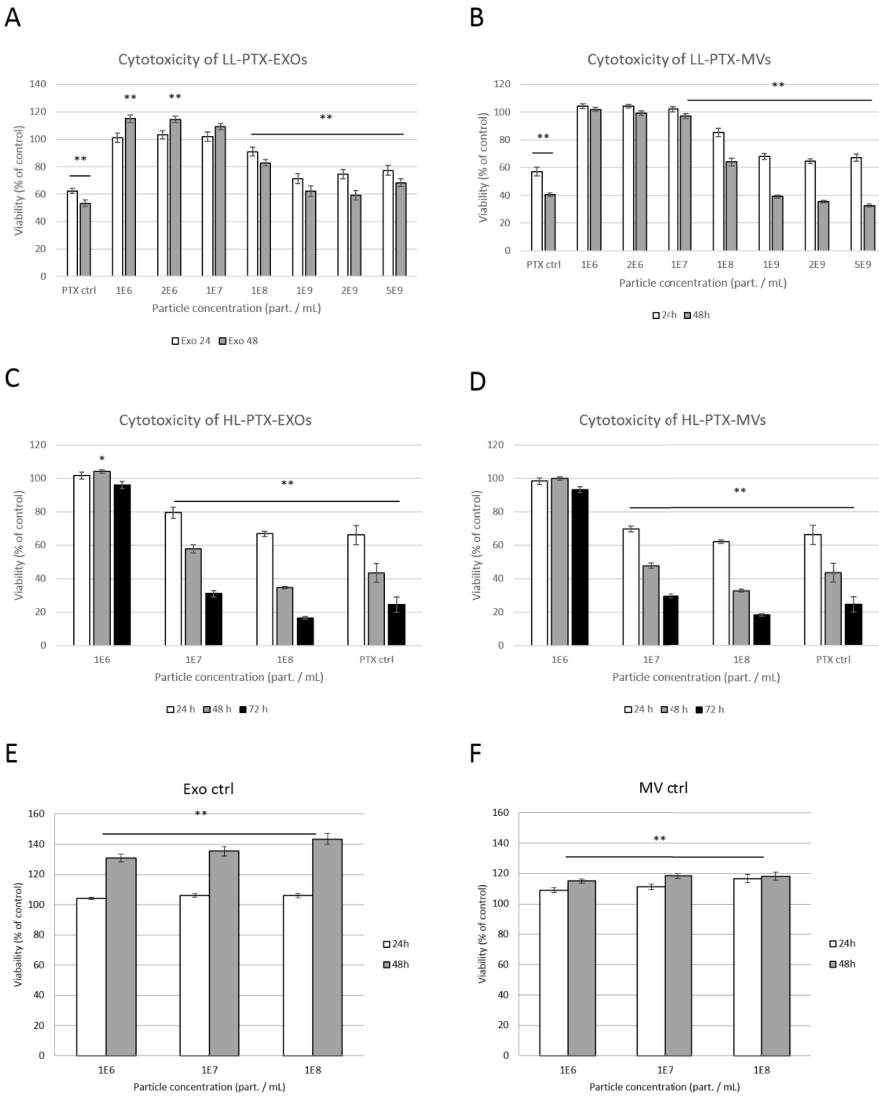


Figure 5: Dose-dependence of EV-mediated PTX delivery. PC-3 derived EVs were incubated with PC-3 cells and viability of the cells was measured as percentage compared to mock control cells after 24, 48 and 72 h. (A and B) 110K EXOs (A) and 20K MVs (B) were loaded with PTX at 1 nmol per 10^9 particles (LL-PTX-EXOs and LL-PTX-MVs)) and added to cells at different particle concentrations with 5 µM PTX as the control. (C and D) the cytotoxicity of 110K EXOs (C) and 20K MVs (D) was assessed after loading them with PTX at 5 nmol per 10^8 particles (HL-PTX-EXOs and HL-PTX-MVs). 20K MVs seemed to more sensitive to the treatment, as was demonstrated by slightly reduced cytotoxicity and uptake rate. (E and F) The effect of 110K EXOs (E) and 20K MVs (F) on cell viability was examined without PTX.

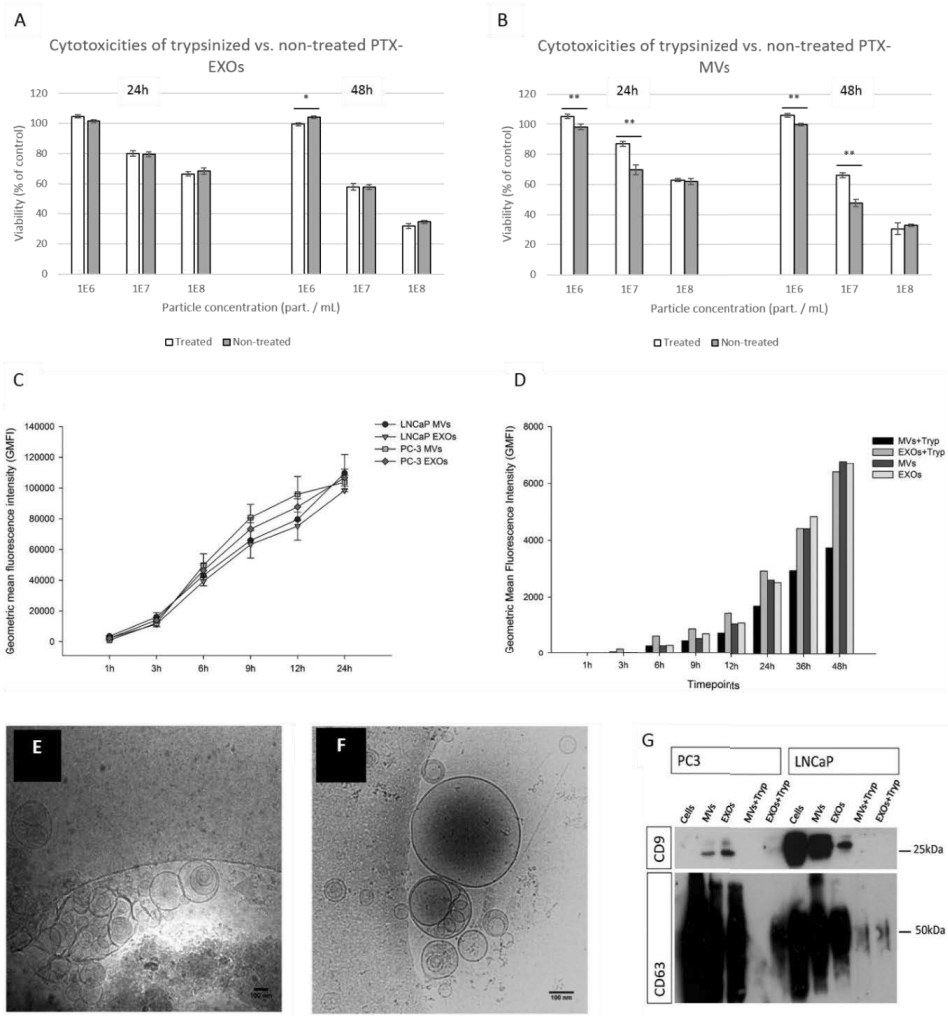


Figure 6: The effect of trypsin treatment on the EVs and their drug delivery properties. (A and B) HL-PTX-EXOs (A) and HL-PTX-MVs (B) were loaded with PTX and their surface proteins were removed by trypsin treatment. They were then added to PC-3 cells and their effect on cell viability was compared to non-trypsinized control EVs. (C and D) PC-3 and LNCaP EVs were labeled with DilC18 (5)-DS and their cellular binding and uptake was measured as a function of incubation time with flow cytometry. In C, EV subtypes from both cells are compared to each other. In D, PC-3 EVs were trypsinized and compared to non-treated controls. (E and F) Cryo-TEM images of 20K MVs before (E) and after (F) trypsin treatment. G: The effect of trypsin treatment on EV surface proteins CD9 and CD63 was assessed by western blotting.

be relevant because of this, since a smaller load of PTX per vesicle was unable to reach its full cytotoxic potential in the case of EXOs, while a higher PTX load was clearly more effective. The surface proteins of EVs appear to have little effect on cellular internalization, though the uptake of

4.2 Extracellular vesicles carry paclitaxel into PC-3 cells via endocytosis and display subtype-dependent differences in their drug release mechanisms

To study the uptake mechanism of PTX-EVs, fluorescently labelled EVs and PTX were used to track their intracellular trafficking with confocal live cell imaging (Figure 7). First, labelled EVs without PTX were observed to co-localize strongly with lysotracker, a dye used to label members of the endosomal pathway, suggesting that EVs were taken up via endocytosis (Figure 7A). EVs carrying fluorescent PTX (PTX-oregon green, PTX-OG) were found to carry PTX-OG with them into the cells with partially similar staining patterns (Figure 7B). It should be pointed out however, that fluorescent labels used to label EVs (DiD) and PTX-OG can form aggregates that co-sediment with EVs during post-labeling washes or leak from the EVs and affect the observed dye distribution, given that they are lipophilic compounds capable of binding and diffusing through the plasma membrane of cells (Dominkuš *et al*., 2018). In addition to the spot-like staining of the EVs, in some cells the PTX-OG had also spread into the cells' cytoplasm and displayed a much higher total fluorescence intensity in the cells, respectfully designated here as "low intensity" (LI) and "high intensity" (HI) cells. While free PTX normally binds to and stabilizes the microtubules, as observed in HI-cells, causing its cytotoxic effect (Singla, Garg & Aggarwal, 2002), the EV-mediated endosomal delivery route seemed to alter the subcellular distribution of PTX in LI-cells, suggesting that the results are not an artefact of leaked or aggregated PTX-OG. In LI-cells PTX-OG was restricted into the endosomal network without compromising its cytotoxic effect and killing the cells within 24 hours. To further elucidate the observed mechanism of EV-mediated PTX delivery, the trafficking of PTX-OG inside PC-3 cells was studied by live cell fluorescence lifetime imaging microscopy (FLIM) (Figure 8). Since fluorescence lifetime is dependent on the immediate environmental conditions surrounding the fluorescent molecule, such as pH, viscosity or polarity, FLIM gives information about the changes of environment that PTX-OG goes through when it is delivered from EVs to cells and until the cells die. Free PTX-OG passed through the cell membrane rapidly and stained the microtubules as shown in Figure 8A. While the staining pattern remained

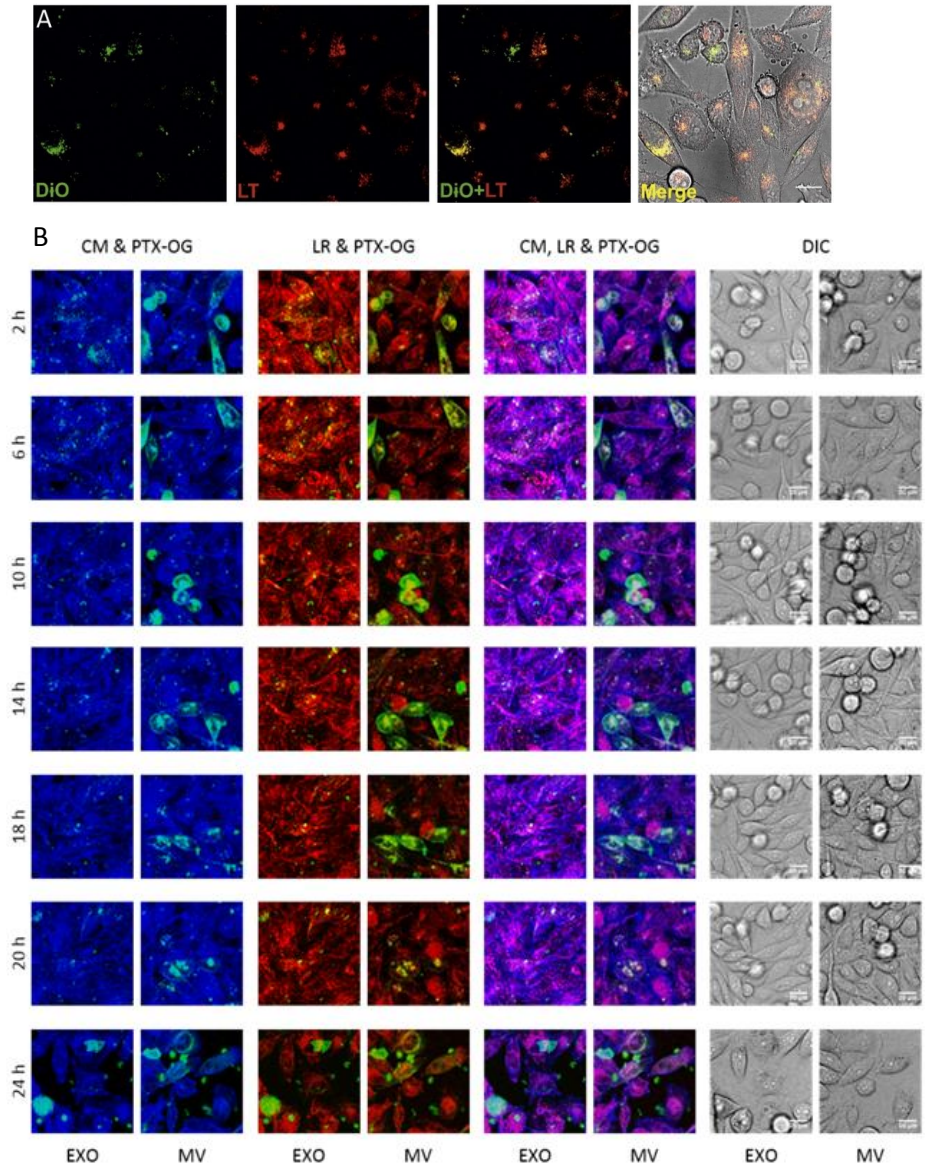


Figure 7: Confocal microscopy analysis of the uptake of EVs and EV-mediated PTX-OG delivery in PC-3 cells. (A) The co-localization of EVs and endo-lysosomal compartments in cells was examined with DiO-labeled EVs and LysoTracker Red (LT), showing significant co-localization of the two dyes after 24 hours. (B) The cellular distribution of EV-delivered PTX-OG (green) was examined every 4 hours after 2 hours of incubation with the PTX-OG-EVs. The cells' plasma membranes were stained with CellMask Deep Red Plasma membrane label (CM, blue) and LysoTracker Red (LR, red).

unchanged throughout the imaging, there was a small change towards shorter average fluorescence lifetime ($<\tau>_\alpha$) after 4 hours of incubation as indicated by a shift to a greener color in the FLIM images. This result suggests a change in the immediate environment of the microtubules, caused by the binding of PTX-OG. PTX-OG carried by EVs displayed two distinct patterns of cell staining and fluorescence lifetime profiles. Cells treated with 110K EXOs carrying PTX-OG were only found to display LI-cells (Figure 8B), while both LI- and HI-cells (Figure 8C) were found in samples treated with the 20K MVs. In HI-cells the staining after 2.5 hours resembled that caused by free PTX-OG (Figure 8A) in pattern, $<\tau>\alpha$ and intensity. LI-cells displayed a completely different pattern, with a shorter $<\tau>\alpha$ and a more spot-like staining in the cytoplasm, instead of the fibril shapes of microtubules. Confocal microscopy imaging revealed that this spot-like pattern corresponded to the endosomal network (Figure7A). Taken together, the PTX-OG in HI-cells appeared to avoid or escape accumulation in the endosomal pathway much faster than in LI-cells, indicating a possibility for an alternative uptake pathway in HI-cells.

Analysis of the fluorescence lifetime signal revealed that it consisted of two components with lifetimes of approximately 4 – 4.3 ns (τ_1) and 1 – 2.7 ns (τ_2). The lifetime each component and their portions of the total fluorescence signal changed over time in the cells as shown in Table 2 and Figure 9. The observed changes could be divided into three phases (1 – 3 in figure 9) for both components: first (1) the fluorescence lifetime rose rapidly during the initial 2 hours of the measurement as PTX-OG-EVs accumulated into the cells. At the peak of the first phase, free PTX-OG and HI-cells had similar, higher fluorescence lifetimes than LI-cells, though the difference was most notable in τ_2. Next (2), the fluorescence lifetime started to decline, for approximately 10 hours, representing a change in the environment of PTX-OG. This phase could be interpreted to reflect the release of PTX-OG from the EVs or the progression of PTX-OG-EVs in the endosomal network in LI-cells and the changes occurring in the microtubules of free PTX-cells and HI-cells. Finally, (3), as the cells died, the fluorescence lifetimes of PTX-OG rose to a similar level in all sample types, signifying that the cellular environment was similar in all apoptotic cells, regardless of the delivery pathway of PTX-OG.

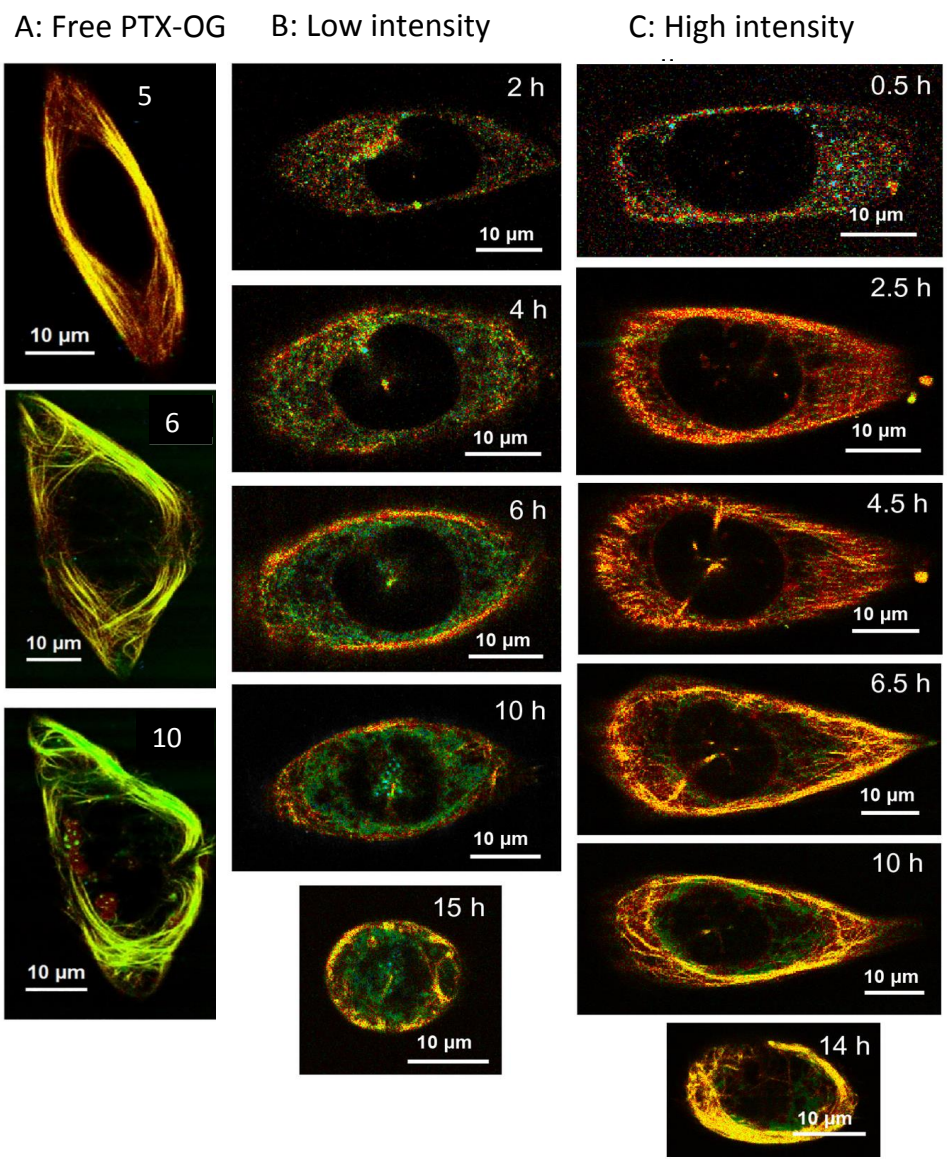


Figure 8: FLIM imaging of PTX-OG distribution in PC-3 cells. Fluorescence lifetime of PTX-OG was measured in cells at different time points after adding free PTX-OG (A), or PTX-OG EVs (B and C). In B, a "high intensity" cell is presented, as identified in 20K MV experiments. In C, a "low intensity" cell is presented, identified with both types of EVs. The color of each pixel in the FLIM images correlates with the average fluorescence lifetime in the corresponding spatial location as follows: 2.0 ns  4.5 ns.

Table 2. Fluorescence lifetime components τ_1 *and* τ_2, the portion a_2 *of* τ_2, and average fluorescence lifetime, $<\tau>_\alpha$, for Ptx-OG in each experimental setup, with HI- and LI-cells separately.

Cell samples	Interaction time	τ_1 (ns)	τ_2 (ns)	a_2 (%)	$<\tau>_\alpha$
Free Ptx-OG in PC-3 cells (*N* = 2)	0–0.5 h	4.3	2.7	28	3.9
	12 h	4.0	1.9	32	3.3
Ptx-OG-20 K MVs in PC-3 HI-cells (*N* = 3)	3 h	4.3	2.3	25	3.8
	15 h	4.0	1.7	27	3.4
Ptx-OG-20 K MVs in PC-3 LI-cells (*N* = 4)	2.5 h	4.2	1.4	35	3.2
	16 h	4.0	1.5	34	3.1
Ptx-OG-110 K EXOs in PC-3 cells (*N* = 4)	2.5 h	4.1	1.2	44	2.8
	16 h	4.0	1.8	30	3.4

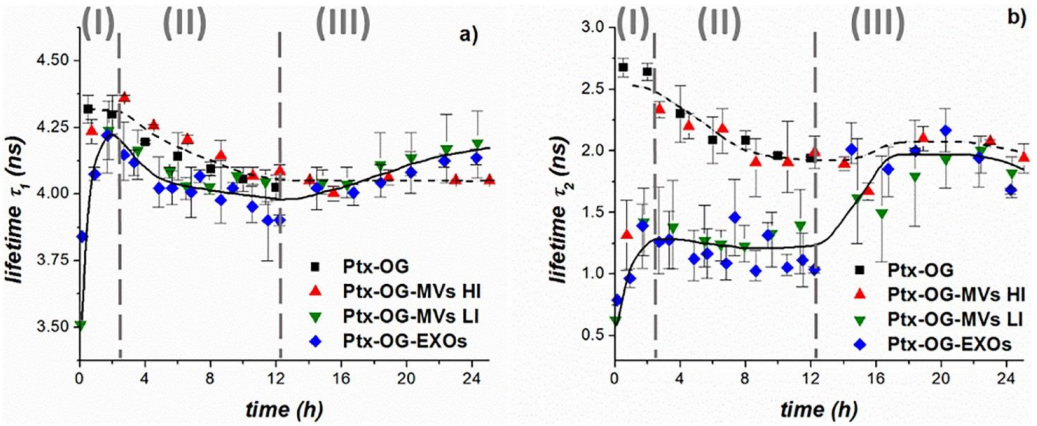
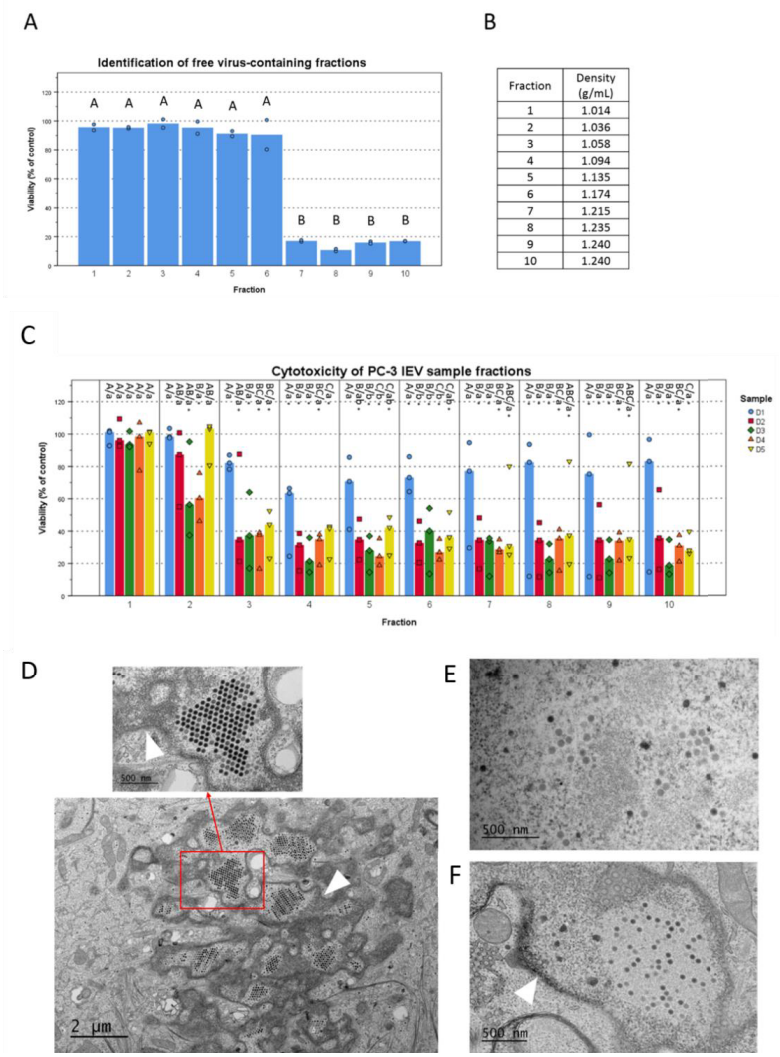


Figure 9: Average fluorescence lifetimes of the two components of PTX-OG fluorescence in PC-3 cells. Fluorescence lifetimes of PTX-OG were measured in cells as a function of time, with average lifetimes for components τ_1 (a) and τ_2 (b) presented separately for each condition.

In summary, FLIM was used to confirm that there was a significant difference in PTX-OG delivery in recipient PC-3 cancer cells caused by the EV-mediated delivery compared to the diffusion of free PTX-OG, as indicated by $\boldsymbol{\tau_2}$. Based on the distribution of $\boldsymbol{\tau_1}$ in cells and its consistency, it appears to reflect PTX-OG bound to the plasma membrane and possibly other cellular membranes, while the value of $\boldsymbol{\tau_2}$ was clearly more dependent on the delivery route and it was also more prominent in LI-cells than in HI-cells. Therefore, $\boldsymbol{\tau_2}$ can be more useful to follow the trafficking of PTX-OG and predict the mechanisms involved, where lower or higher values could indicate an environment corresponding to the endosomal network or values binding to microtubules, respectively.

4.3 Extracellular vesicles as carriers of oncolytic adenovirus

Next, it was examined whether EVs isolated from cancer cells infected with an oncolytic adenovirus could be used as carriers of the virus, and if they displayed any significant differences compared to normally secreted EVs. For this purpose, A549 and PC-3 cancer cells were infected with Ad5/3-D24-GMCSF virus (Koski *et al*., 2010) and the EVs were collected and purified every 24 hours for five days (D1 – D5) by iodixanol gradient centrifugation. As the results from both cell lines were nearly identical in most experiments, only the data from PC-3 cells is presented in such cases. The density fractions from D1 – D5 were analyzed for cytotoxicity, nanoparticle-, total protein-, adenoviral hexon surface protein- and adenoviral DNA concentrations to identify and characterize the EVs from infected cells (IEVs) for viral cargo and its functionality. Given that EVs have a characteristic range of density that is lower than the density of adenoviruses (1,21 g/mL) in iodixanol (Dormond *et al*., 2010), IEVs were expected to be separated effectively from any free virions present in the cell culture supernatant into fractions of lower density. Cytotoxicity assay of the density fractions revealed that virions isolated from infected cells indeed migrated to the expected density in iodixanol gradient (fractions 7 – 10) (Figures 10A and B). However, in addition to these virion-containing fractions, with IEV samples also lighter fractions (2 – 6) were found to be cytotoxic (Figures 10C and D). TEM imaging of the cells treated with fraction 4 revealed that they did contain virions in their nuclei, confirming viral infection caused by the IEV fraction (Figures 10E – G). The most striking observation, however, was that the infective IEVs appeared as early as one day after the initial infection (D1), which was prior to the lytic release of adenoviruses, denoted as the classical pathway that the adenovirus uses to spread (Gros, Guedan, 2010). As shown in figure 11, after being infected with the virus, the cells start to show early signs of apoptosis only after D2, leading to virtually all of the cells dying by D5.



Fraction	Density (g/mL)
1	1.014
2	1.036
3	1.058
4	1.094
5	1.135
6	1.174
7	1.215
8	1.235
9	1.240
10	1.240

Figure 10: Cytotoxicity assessment of IEV fractions. (A) The buoyant density of free virions isolated from cells was determined with a cytotoxicity assay from each fraction with A549 cells. Only fractions 7 – 10 were found to by cytotoxic. (B) Table of densities for each fraction collected. (C) cytotoxicity of IEV iodixanol density fractions. IEVs were collected from PC-3 cells every 24 hours for five days (D1 – D5) after initial infection and purified with iodixanol density gradient. Cytotoxicity of each fraction was determined with autologous cells with an equal volume from each fraction. (D – F) TEM imaging of A549 cells after 48h with IEV D3 fraction 4 (D), PC-3 after 24h with IEV D3 fraction 4 (E) A549 after 48h with IEV D1 fraction 4 (F). White markers indicate the nuclear membrane of the treated cells, containing hexagon-shaped adenoviral virions.

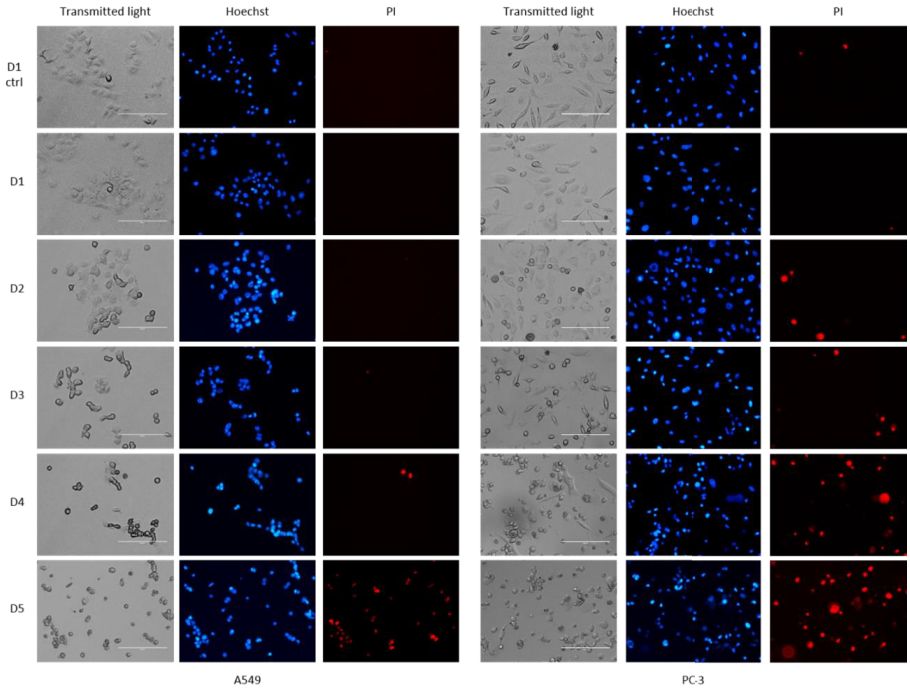


Figure 11: Progress of apoptosis in infected cells. The progress of apoptosis in A549 and PC-3 cells during D1 – D5 was monitored by fluorescence microscopy using Hoechst 33342 and propidium iodide (PI) staining. Nuclear condensation is observed earliest at D2 suggesting early apoptosis, leading to cell rounding at D3 and permeation of PI at D4 to D5, which are markers for late apoptosis.

The distribution of IEVs in the gradient corresponded to the normal EVs secreted by these cells as indicated by NTA assay of the fractions (Figure 12A). The IEV and EV fractions contained also comparable amounts of protein per particle (Figure 12B) and they had similar size distributions (Figure 12C), suggesting no significant differences in their basic structures. Cryo-TEM imaging of the IEV fractions showed vesicle morphology typical for EVs (Figure 12D), with no evidence of virions inside or co-purified in the samples from 357 images analyzed. Analyzing the

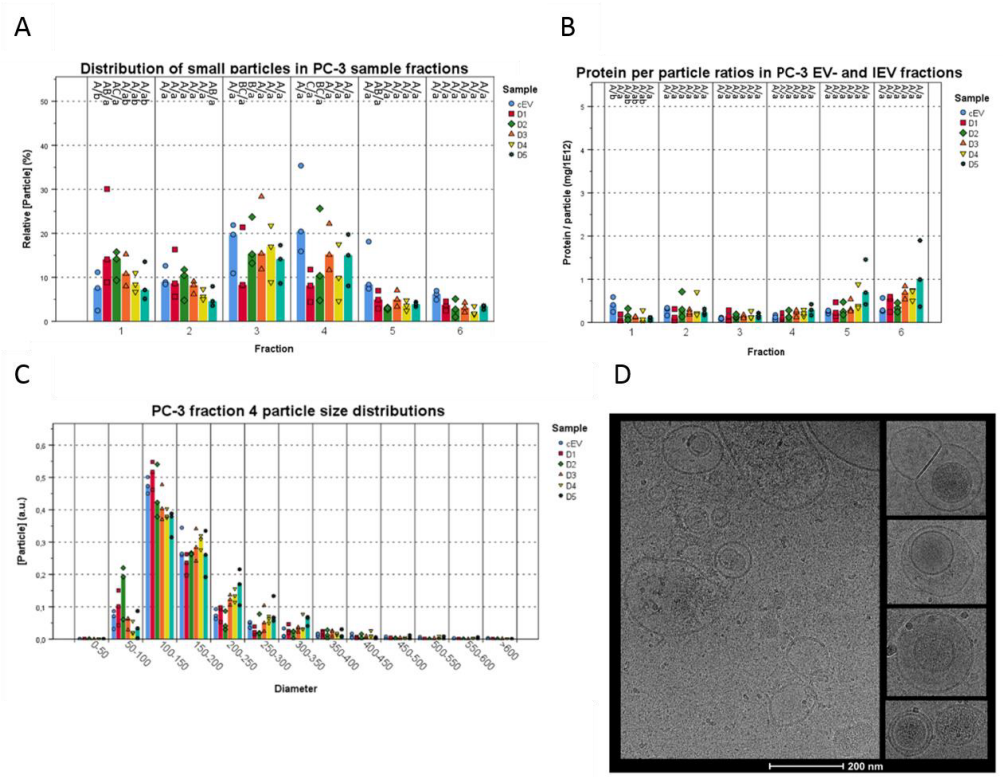


Figure 12: Physical characterization of PC-3 IEVs. IEV fractions 1 – 6 were analyzed with NTA and total protein assay to determine relative particle concentrations (A) and protein per particle ratios (B) in each fraction and the size distribution of particles, with the result of fraction 4 as a representative fraction (C). Cryo-TEM imaging was used to visualize IEVs collected after five days of uninterrupted IEV secretion in cells (D), showing vesicle structures typical to EVs.

samples with anti-adenoviral antibodies still indicated a presence of viral proteins in the IEV samples (Figure 13). A dotblot analysis of each fraction from D1 – D5 (Figure 13A) showed very little or undetectable amounts of adenoviral hexon protein in D1 and D2 samples, with a significant increase across all fractions after D3, following the apoptotic cell lysis. Hexon protein was present in the IEV fractions with a consistent peak at fraction 4, though at lower concentrations than in the free virus fractions 7 – 10. Western blot analysis of IEVs with a polyclonal anti-adenovirus antibody showed that compared to infected cells, IEVs are enriched in hexon over other viral proteins (Figure 13B). Incubating IEVs with this antibody also neutralized

their infectivity completely (Figure 14A), suggesting that the proteins on the outer surface of IEVs are critical to their function. Also, unlike free virions, IEVs were found to be sensitive to detergent treatment, again losing their infectivity (Figure 14B), indicating that the infectivity of IEVs is independent of viral capsids that would endure detergents.

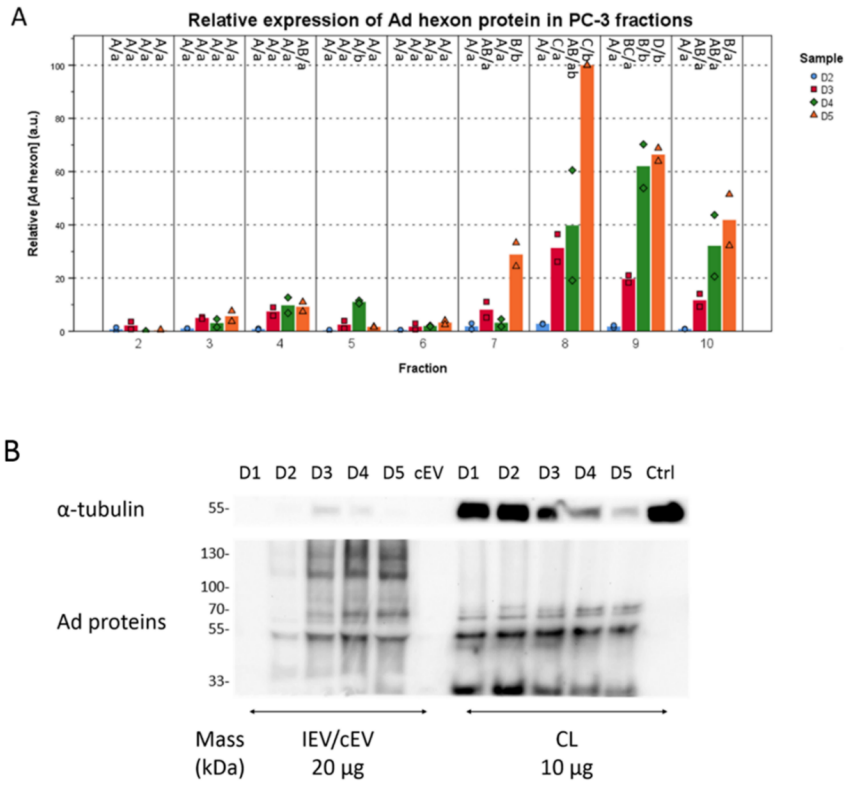


Figure 13. Characterization of viral proteins associated to PC-3 IEVs. (A) The expression of adenoviral hexon protein was assessed in PC-3 IEV fractions from all time points D1 – D5 by dot blotting and normalized against the highest expression level, usually at fraction 8 of D5. D1 samples did not produce a high enough signal to be measured. (B) The viral protein composition of pooled IEV fractions (1 – 6) from each time point was compared to corresponding cell lysates by western blotting with a polyclonal anti-adenovirus antibody, with α-tubulin examined as a housekeeping protein. Compared to cell lysates, IEVs start to express observable amounts of viral proteins after D2 and they are enriched with hexon protein (110 and 130 kDa bands). In cells, the expression of viral proteins also increased over time, as indicated by a relative drop in α-tubulin expression.

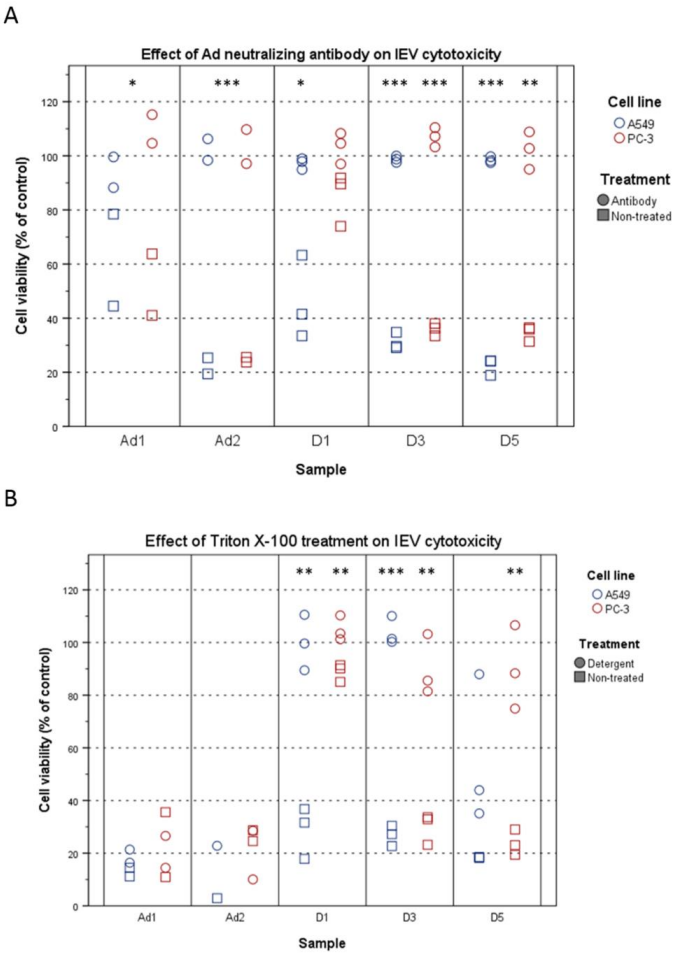


Figure 14. IEV-mediated infectivity is sensitive to adenovirus neutralizing antibody and detergent treatment. IEVs and adenovirus control samples (Ad1 high and Ad2 low concentration) were treated with an adenovirus neutralizing antibody (A) or detergent (B), followed by a cytotoxicity assay. Compared to non-treated controls, IEVs lost their infectivity in almost all of the cases (A549 D5 samples partially survived the detergent treatment), while adenovirus controls were only neutralized by the antibody.

The adenoviral DNA content of IEVs and other fractions was also analyzed by qPCR (Figure 15), confirming the presence of viral DNA in the IEV fractions 1 – 6 in addition to the free virus fractions (7 – 10) (Figure 15A). The distribution of viral DNA across the IEV fractions appeared to be dependent on fraction density, as fraction 6 contained the most viral DNA of all IEV fractions,

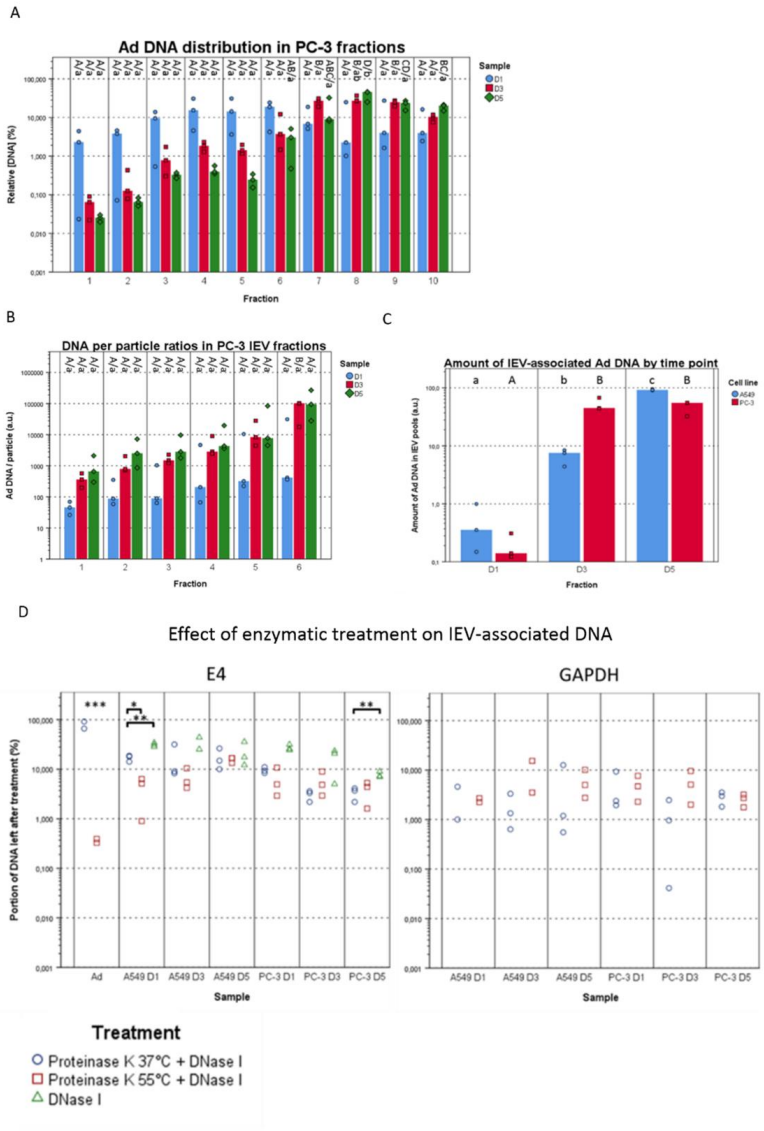


Figure 15. Characterization of IEV-associated adenoviral DNA. The concentration of adenoviral DNA was estimated with RT-PCR in A – D, with primers against adenovirus *E4* gene (or human *GAPDH* for comparison in D). Results with *E1A* primers provided near identical results. (A) The adenoviral DNA concentration was assessed in all PC-3 sample fractions from different time points and normalized for each time point separately to assess its distribution across the gradient. (B) The amount of adenoviral DNA was compared to the particle concentration in PC-3 IEV fractions to estimate DNA per particle ratios. (C) The total amount of IEV-associated adenoviral DNA was calculated for different time points in pooled fractions 1 – 6 and

normalized against the highest concentration. (D) Adenoviral virions (Ad) and IEVs were treated with proteinase K and DNase I in different conditions to assess the localization of their DNA content. Treatments were performed with or without proteinase K at +37 °C or +55 °C to cleave shielding proteins, followed by DNase I treatment to digest the DNA. The results are reported as remaining DNA compared to pre-treatment concentrations.

which was contrary to hexon and particle distributions that had peaks at fraction 4 (Figures 12A and 13A). This relationship was also apparent when the viral DNA concentration was compared to the particle concentrations, showing that particles with higher density were associated with more DNA (Figure 15B). The viral DNA was also shown to contain near equal amounts of genes *E1A* and *E4*, located at the opposite ends of the adenoviral genome, suggesting that the whole genome was present in IEVs. The total amount of viral DNA in the IEV fractions increased along the progression of infection from D1 to D5 (Figure 15C) and like cellular DNA in the IEVs, it was partially resistant to the enzymatic treatment used to cleave DNA in free virions (Figure 15D). Approximately 70 – 95 % of the viral DNA associated with IEVs was degraded as a result of different variations of the proteinase K treatment followed by DNase, suggesting that most of the DNA was bound to the outer surface of IEVs. Compared to free virions, which required proteinase K treatment at +55 °C to release their DNA for degradation, in IEVs the differences between treatment temperatures of 37 °C to +55 °C or even total absence of proteinase K did not cause a large difference in DNA degradation. Taken together, these results indicate that the DNA associated to IEVs is not protected by a durable protein capsid as Ad virions have, but most of it is exposed on the outer surface of the vesicle and rest of it is inside, where it is protected from both the enzymes, even at +55 °C.

Taken together, cancer cells infected with an oncolytic adenovirus secreted IEVs that carried viral cargo and were able to transmit it to neighboring cancer cells, spreading the infection. These IEVs were secreted already at the early stages of the infection, before the apoptotic release of virions, and the amount of viral cargo they carried reflected the stage of the infection. Additionally, while IEVs were similar to the normal EVs secreted by these cells in their physical properties, some density related heterogeneity existed among the IEVs, with more viral DNA being associated with the heaviest vesicles.

5 Discussion

5.1 Autologous cancer cell-derived extracellular vesicles are effective as carriers of chemotherapeutic cargo

In this thesis, the use of EVs from different cancer cells as an autologous delivery system was examined with PTX and oncolytic adenovirus as the model therapeutic cargo. The hypothesis was that since cancer cells use EVs to communicate between each other, the therapeutic cargo of EVs would also be internalized by the cells during EV uptake. This kind of autologous drug delivery approach could help circumvent the problem caused by the heterogeneity of tumors, the fact that a single drug targeting moiety may not recognize all of the cancer cells of a tumor because they express different phenotypes. Additionally, cancer cells provide a pathway for the preparation of oncolytic virus-carrying IEVs that cannot be applied with normal cells without modifying the virus or the cells by genetic engineering, since oncolytic viruses by definition cannot replicate in healthy cells. In these studies, all of the PTX-EV or IEV preparations were found to be functional, i.e. they were able to carry the cargo into the cancer cells and cause a cytotoxic effect. In the case of PTX-loaded EVs, the cytotoxic effect of PTX was even enhanced, when the ratio of PTX per EV was optimized. This enhancing effect has been reported by others as well, with the use of different drug loading methods and drugs (Kim *et al*., 2016, Tang *et al*., 2012, Yang *et al*., 2015), adding to the accumulating evidence for EVs having potential drug delivery capabilities. However, as found here and by others, autologous cancer cell-derived EVs also possess an innate viability enhancing effect in recipient cancer cells (Ozawa *et al*., 2018, Raimondo *et al*., 2015), as well as other cancer promoting effects discussed in section 1.1.4. This viability enhancing effect is exactly the opposite to the desired effect of killing cancer cells, and makes direct applications based on cancerous EVs unsafe in practice. In future it might be possible to neutralize the cancer promoting cargo of these EVs by e.g. removing it through temporary pores by methods described in section 1.2.2 or by inactivating it by ionizing radiation (Eldh *et al*., 2010). Neutralizing the cargo would, however lead also to the loss of any biological therapeutic molecules, such as Ad DNA or anti-cancer RNAs. Additionally, cultivating a patient's cancer cells for *ex vivo* EV-production poses a formidable challenge in itself. Therefore, the best way to take advantage of the cancer cell-derived EVs in practice is to learn from them and mimic them: identify the most critical surface structures and

reproduce them in a well-characterized, safer EVs for cancer targeted delivery. In study (I), it was shown that trypsinization of the PTX-EVs was able to diminish their cytotoxic effect only partially, suggesting that the EV surface proteins are not critical for their uptake, and that the cells must also be able to bind to something else in their lipid membrane. Glycolipids represent a strong targeting candidate given that heparan sulfate competitively inhibits the uptake of most EVs (Atai *et al*., 2013), blocking their glycans from interacting with the cell surface, although other glycan structures besides heparin sulfate, such as those in ganglioside GM2 have been identified as ligands in lipid-protein signaling (Kundu *et al*., 2016), and PS has its own receptors as well (Segawa, Nagata, 2015).

While cell binding followed by EV uptake in general is crucial for the delivery of any cargo, subcellular trafficking of the cargo may also be important regarding its activity in the recipient cell. It has been shown that the protein content of EVs separates the lipids into the lysosomes from once inside the cell (Tian *et al*., 2010). However, it is currently unclear, if there are any subcellular targeting moieties on the EVs that would guide them into specific cellular organelles besides the endosomal network. To release any intraluminal cargo, the internalized EVs would either have to fuse with the endosomes, or have their membrane degraded. Any cargo that is bound to the outer surface of the EVs would also have to detach from the EVs in the endosome in order to reach its relevant subcellular destination. In any case, the EVs would lose their structure and cargo, with their components being either degraded or sorted into their respective subcellular destinations. Therefore, subcellular targeting of their cargo should be possible by modifying the cargo molecules themselves as described by Sakhrani and Padh (Sakhrani, Padh, 2013). In studies (I) and (II), the PTX-OG in PTX-OG-EVs was shown to be internalized into the endosomal pathway in LI-cells and to bind to the microtubules in HI-cells, killing the cells regardless of the final subcellular destination of the drug. This observation of different localization of PTX-OG is contrary to the main reported mechanism for PTX cytotoxicity, which is based on stabilizing microtubules inhibiting depolymerization. The contradiction could be explained if PTX bound to and inhibited also another target besides microtubules. It has been shown that PTX can bind to apoptosis regulator Bcl-2 (Ferlini *et al*., 2009), located on the membranes of mitochondria, endoplasmic reticulum and nuclear membrane (uniprot database). Given that the endosomal trafficking also occurs to the trans-Golgi network, it would enable PTX to spread into virtually any cellular organelle through intracellular vesicle trafficking (Elkin, Lakoduk & Schmid, 2016). In any

case, PTX-OG was shown to both follow the endosomal network in the LI-cells and to escape it within 2.5 hours in the HI-cells, suggesting that the sorting of EV cargo may be altered in HI-cells, or that certain EVs that behave differently were enriched into them. It has been suggested that the cell cycle phase of the recipient cells affects their EV internalization (Lazaro-Ibanez *et al*., 2017), however there must also be some difference between 20K MVs and 110K EXOs, since HI-cells were only observed in the former. As presented in study (II), FLIM provided a powerful addition to the methods for studying EV intracellular trafficking, giving more detailed information about the cellular environment of PTX-OG, than conventional fluorescence microscopy methods. In future studies, FLIM can be used to determine the changes in environment that EVs go through, and what effects do these changes have regarding the fate of EVs in cells.

5.2 Extracellular vesicles divided by their density into subtypes have different cargo profiles and delivery mechanisms

The 20K MV fraction used in the experiments, where HI-cells were identified with PTX-OG staining, was separated from 110K EXOs based on sedimentation speed. Given that the size distributions of these two populations were overlapping, the higher sedimentation speed of 20K MVs suggests that they have a higher density. From the cryo-TEM images of 20K MVs it can be determined that most of the vesicles are multilamellar, i.e. they contain more than one lipid bilayer. The apparent enrichment of multilamellar EVs in the 20K MV samples would explain the increase in density and possibly also the decrease in protein per particle ratio that was reported in both studies I (Figure 1E) and II (Figure S3), caused by reduced space for soluble cargo. The same explanation may also be related to the observed reduced viability enhancing effect in study I (Figures 4 and 5), when comparing 20K MVs to 110K EXOs, if the latter contained more soluble cargo, including different RNA species, metabolites and soluble proteins. The western blot analysis of these two sample types in figure 1F seems inconclusive though, showing no apparent trend regarding soluble or membrane protein enrichment in either population compared to the other. Multilamellar EVs should also have an increased capacity to bind hydrophobic compounds such as PTX, though again no notable differences could be observed in study (I). While the hypothesis of enrichment of multilamellarity in 20K MVs still seems possible, it requires validation in future studies and its implications to cargo delivery are difficult to predict. If the release of EV cargo in the endosomes is

dependent on the fusion of the EV outer membrane with the endosomal membrane, then in the case of multilamellar EVs, it would lead to the release of their inner vesicles into the cytoplasm, where their concomitant fate remains unknown. Similar heterogeneity with EVs isolated using a similar protocol to ours, has been reported earlier in a study by Kanada *et al.* (2015) (Kanada *et al*., 2015). In this study, MVs were described as significantly more effective carriers of siRNA, mRNA and DNA that were transiently expressed in the EV producing cells, partially because they carried more of these molecules than the corresponding EXOs. The results also suggested that the nucleic acid cargo of EVs was primarily degraded in the endo/lysosomal compartment, leading to no gene silencing or expression except for plasmid DNA delivery. Since functional EV-mediated delivery of mRNA and other RNA species has been reported elsewhere (Alvarez-Erviti *et al*., 2011, Ohno *et al*., 2013, O'Brien *et al*., 2015), the negative results in the study by Kanada *et al.* may be due to cell line-specific differences in the EV internalization mechanisms. Certain EVs have been shown to be dependent on caveolar endocytosis, which is an endocytic pathway that does not necessarily lead to degradation by fusion with lysosomes, the classical end point in other pathways (Kiss, Botos, 2009). EVs from Epstein-Barr virus-infected cells for example have been shown to be dependent on caveolin-mediated cellular uptake (Nanbo *et al*., 2013). Targeting caveolar endocytosis might therefore be a viable strategy for improving EV-mediated functional transfer of biomolecules, avoiding their degradation. Caveolae and clathrin-mediated endocytosis as well as micropinocytosis have also been reported to mediate transcytosis, where cargo passes through the internalizing cell (Elkin, Lakoduk & Schmid, 2016, Matsumoto *et al*., 2017). Transcytosis has been suggested as a possible mechanism for EVs to cross biological barriers, such as the blood-brain-barrier, which normally inhibits the crossing of large particles and hydrophilic drugs to the brain (Matsumoto *et al*., 2017). Therefore, targeting transcytosis could be a beneficial strategy for EV-mediated drug delivery to the brain and other restricted tissues.

In study (III), no separation of EV subpopulations was performed by differential centrifugation, so similar comparison between 20K MVs and 110K EXOs is not available. However, density-related differences were observed in the IEVs, with heavier IEVs containing more protein and viral DNA per particle. The amount of viral DNA was also reflected in the functionality of IEVs, in that the lightest IEVs displayed reduced cytotoxicity, when compared to the heavier IEV fractions. Therefore, this reduced cytotoxicity can be explained by an insufficient amount of viral DNA for infection. Nuclear import of naked, exogenous DNA in cells is not very efficient due to its

degradation and slow migration through the nuclear pore complex (NPC) (Dean, Strong & Zimmer, 2005, Lechardeur *et al*., 1999, Salman *et al*., 2001, Bai *et al*., 2017), which would imply that a considerable amount of DNA needs to be endocytosed in order for it to be imported into the nucleus. Because cells can be saturated by EVs as was observed in study (I) and by others (Heusermann *et al*., 2016) and therefore their rate of uptake is limited, the concentration of cargo per vesicle can determine if the cargo's function will be fulfilled. As a simplified case of degradable biomolecular cargo such as DNA in cells for example, a critical concentration in the cell required for effective nuclear delivery cannot be reached via EV-mediated delivery if the rate of DNA intake is not greater than the rate of DNA degradation prior to nuclear transport. Therefore, due to this and the saturation effect, EVs with too small DNA per vesicle content could not cause an effective transfection of the recipient cell, which was observed regarding fraction 1 IEVs in study (III), even though they did contain viral DNA as well, and in theory the nuclear uptake of only one copy could lead to a successful infection. In contrast to EVs, the mechanism of virion-mediated adenoviral DNA import protects the DNA during intracellular transport. The viral DNA is carried to the NPC still packed in the viral capsid, from where it is then directed into the nucleus through the NPC, and therefore remains shielded from the DNA degrading cytoplasmic nucleases (Fay, Panté, 2015). For this reason, EV-mediated viral DNA delivery can be less effective if it is based on releasing the DNA content unprotected into the cytoplasm. However, this mechanism remains unknown at this point and it may also vary between different EV types and DNA sequences. For example, some DNA sequences act as guides for nuclear transport that improve transfection efficiency (Sebestyen *et al*., 1998) and in theory could be enriched in EVs for a more effective nuclear delivery.

5.3 Extracellular vesicles act as alternative carriers of oncolytic adenovirus genome to non-enveloped virions

EVs are more flexible as therapeutic delivery agents for viral DNA regarding the initial cell recognition and cellular uptake, for which virions depend on the presence of a specific receptor on the cell surface. For this reason, IEVs are able to infect cells that are lacking the receptors required by a free virion (Ran *et al*., 2016). Some IEVs have also been shown to be resistant to opsonization by antiviral antibodies and overall innate antiviral immune response that is triggered by the recognition of virions by the immune cells (Ran *et al*., 2016, Maguire *et al*., 2012). This was not the

case in study (III), as the IEVs were completely neutralized by adenovirus recognizing antibody, which means that they would most likely also induce a similar immune response as free virions. The results also indicated that an entire capsid is not present inside the IEVs, but instead the vesicles are coated with viral proteins and DNA, which are able to mediate the infection like a whole virion would. Optimally, from a therapeutic perspective, if an entire adenovirus were encapsulated inside an EV, it should retain the efficient intracellular DNA transport mechanism that is characteristic to the virus, while also attaining the cellular targeting and immune evasion properties of EVs. Encapsulation of virions would naturally be restricted to EVs that are larger than the virion (>100 nm in diameter in the case of adenoviruses), but any size dependence should not apply to the IEVs described in study (III), though it was not specifically evaluated. It should also be noted, that the IEVs are a naturally occurring phenomenon and not something artificially induced such as PTX-EVs in study (I). The enrichment of hexon protein to IEVs especially might indicate that the formation of IEVs is intentional for the spreading of the virus, since hexon is the protein responsible for transporting the viral DNA into the nucleus and probably the most important one in this regard to be included in the IEVs (Smith *et al*., 2008). Therefore, any medical trials studying oncolytic viruses for cancer treatment also induce the production of IEVs in the treated cancer cells and they are already partially involved in the observed therapeutic outcomes. Given that clinical trials involving oncolytic viruses rarely show complete tumor eradication, naturally occurring IEVs are likely not effective enough to kill all cancer cells in the patients. While the exact contribution of IEVs is difficult to predict, future studies regarding oncolytic virotherapy should also start focusing on them, by for example optimizing the virus by genetic engineering to increase the loading efficiency of its genome into IEVs by using guiding sequences that target nucleic acids to EVs, inducing the incorporation of useful proteins for improved cell targeting and immune response against cancer. These efforts of virus engineering could also be combined with engineering IEVs for optimal intravenous administration by improving their lifetime in circulation and cancer targeting.

Cancer immunotherapy is another promising approach for treating cancer, where cancer cells are only the indirect target of the treatment, as the primary focus is the antigen presenting cells that are activated against cancer cells. IEVs could also be useful in this approach, given that they also possess direct anti-cancer activity, as compared to plain cancer-derived EVs that have been assessed for cancer immunotherapy applications. The underlying mechanisms of

successful oncolytic virotherapy have been suggested to involve activation of an anti-tumor immune response by the presence of the virus in the tumor, besides the direct killing of infected cancer cells (Lawler *et al*., 2017). In this regard, IEVs carrying both viral and cancer-derived antigens could prove as a powerful anti-cancer vaccine, where the generation of an immune response against cancer antigens could be boosted by the presence of viral antigens that contain pathogen associated molecular patterns readily recognized by immune cells. A similar approach has been proposed with oncolytic viruses carrying peptides derived from cancer cells, which has shown promising results in generating a stronger anti-cancer immune response (Capasso *et al*., 2015).

While it was not in the main scope of this thesis, it cannot be disregarded that besides the therapeutic opportunities of IEVs, they also represent a novel paradigm in virology as alternative vectors for viral genome. As virions are defined as protein- or lipid enveloped particles containing viral genome with the ability to transmit infection, adenovirus-derived IEVs described in study (III) also fit this definition as an enveloped virion, even though adenovirus is a non-enveloped virus. This is especially contradictory if IEVs do not even contain the adenoviral capsid and the IEV-associated DNA seemed to be mostly susceptible to enzymatic cleavage, suggesting that it was located on the outside of IEVs, therefore functioning with a different mechanism to achieve viral infection in the recipient cells. Adenoviral "naked" virions and IEVs would both represent virions of the same species with completely different structures and functionality, different sides of the same coin so to say. The classical virion is much more stable and therefore more useful in transmitting infection from host to host, while IEVs may be useful in overcoming restrictions regarding cell heterogeneity and immune response of the host. In this case, adenovirus could be described as a virus that is able to produce both non-enveloped and enveloped virions. Future studies regarding the enveloped virions of classically non-enveloped viruses can provide important new knowledge about how viral infections spread and how to prevent them, as this phenomenon appears to be a part of the life of other non-enveloped viruses as well.

5.4 Biocompatibility, dosing and purity of extracellular vesicles needs further clarification

Naturally, the autologous origin of EVs would also entail that EVs taken from a healthy individual would be tolerated completely without side-effects, when returned to the body as a form of therapy. In other words, autologous EVs should have optimal biocompatibility in patients, with the exception of EVs derived from cancer cells, infected cells or other EVs that promote the spreading of diseases. Optimal biocompatibility could be a major advantage in the case of chronic diseases that require long-term treatment compared to some synthetic drug carriers, against which the patient may develop sensitivity and whose components may accumulate in the liver and cause toxic effects (Cho *et al*., 2009). Also, autologous EVs should be recognized as "self" by the immune system, leading to no adverse immune responses, which can occur for example with viral vectors (Lawler *et al*., 2017). While the clearance of EVs by the mononuclear phagocyte system remains an issue, if EVs are indeed found to be well tolerated, one might also consider it to be feasible to simply saturate the circulation to circumvent the issue of rapid clearance, if their cargo is equally harmless in bystander cells. This would at the same time override any need for specific targeting by relying on a sufficient number of EVs reaching the target cells passively. This approach could provide an advantage in cases where specific targeting is difficult, such as cancer cells. High doses of EVs are still impractical due to their costly and laborious large-scale production and purification. Given that EVs are non-living biological entities, their maximal applicable dosage should be relatively high, but this remains to be firmly established. However, in a study by Smyth *et al*. (Smyth *et al*., 2015) it was found that injecting mice with 400 µg of EVs as measured by their protein mass, caused asphyxiation due to EV aggregation in the lungs. As this phenomenon appears to be the only harmful side-effect of EVs reported so far, the accumulation of EVs in the lungs could become a considerable indicator for negative effects in future studies regarding the question of EV dosage.

The use of autologous EVs becomes more complicated, when considering EVs from patients with certain diseases, as EVs may also mediate the harmful effects of diseased cells. It may also be too impractical to collect or cultivate donor cells for the production of sufficient EV doses. An alternative option would be to use EVs from healthy donors, such as blood donors or EVs produced in cell lines. The immunological compatibility of EVs from non-autologous human

sources has not yet been systemically evaluated. Studies have been performed so far with for example mesenchymal stem cell-derived EVs for the treatment of a patient with graft-versus-host disease (Kordelas *et al*., 2014). Based on the results, non-autologous human EVs appear to be safe to use even with patients in such critical condition. However, it should be noted that recipients of donated organs are under immunosuppressive medication, which makes it impossible to draw firm conclusions regarding the true immunological compatibility of EVs. Clinical trials have also been performed with EVs for the treatment of cancer, using dendritic cell- or ascites-derived EVs for lung cancer (Morse *et al*., 2005), melanoma and colorectal cancer (Escudier *et al*., 2005, Dai *et al*., 2008) immunotherapy. These studies have also reported good tolerance of the treatment and induction of antitumor immunity that increased the survival time of some patients.

Another important factor regarding the preparation of clinically relevant EVs is that currently there are no methods for establishing their level of absolute purity. It's a very challenging task to determine any universally applicable parameters for EV purity, since the EVs are seemingly all different in composition. Generally, EV purity has been evaluated by such methods as immunoblotting of EV-associated proteins to evaluate their enrichment, calculating protein-to-particle-ratios using total particle- and protein assays (Webber, Clayton, 2013, Nordin *et al*., 2015) and size exclusion chromatography (Nordin *et al*., 2015, Huang *et al*., 2015) or field flow fractioning (Sitar *et al*., 2015) to evaluate the presence of small, non-EV-associated proteins. Recently there have also been reports of using colorimetric (Osteikoetxea *et al*., 2015) and optical methods such as Fourier-transform infrared spectroscopy (Mihaly *et al*., 2017) and Raman spectroscopy (Gualerzi *et al*., 2019) that can be useful in determining protein to lipid ratios in EV preparations that may be used to represent the purity of EVs. While these methods cannot be used to assess absolute values for purity, they could be used in quality control to estimate reproducibility between preparations. Each method has its limits, as immunoblotting may be sensitive to protein isotypes and the specificity and enrichment of different EV markers vary according to the source of EVs, total particle concentrations are difficult to measure reliably (Vestad *et al*., 2017), methods relying on separation by size are sensitive to large contaminants and optical methods are affected by non-specific interference from certain contaminating molecules. A combination of these methods will be likely to help overcome their individual limitations and help determine the purity of EV preparations from different angles. In studies (II and III), density gradient centrifugation was used to purify EVs, which is currently considered one

of the best methods for EV purification, whereas in study (I), only differential centrifugation was used. Most likely the preparations in study (I) did contain contaminating proteins as well as EVs, but the results from fluorescence microscopy were comparable to study (II) and the protein per particle-ratios in both studies had the same trend of 20K MVs having less protein per particle than 110K EXOs, though the exact values are not comparable due to changes in the NTA setup used between those two studies. The protein per particle values between studies (II) and (III) were also very similar, although the values were higher in the high density fractions 5 and 6 in study (III). In study (I), trypsinized PTX-EV samples, where any contaminating proteins would have been cleaved by the enzyme, were also examined with little observed effect on the cytotoxicity, compared to non-trypsinized, where contaminating proteins were left intact. Taken together, these results suggest that contaminating proteins did not affect the results regarding PTX delivery in study (I) in a significant manner. The presence of contaminants effects general EV characterization the most, such as western blotting, size distribution and protein per particle analyses, but the magnitude of the effect remains unclear in study (I) regarding those characteristics, as the results were still comparable to those in studies (II) and (III).

6 Conclusions

EVs provide a versatile platform for drug delivery, with capacity for carrying both small molecule drugs and biological therapeutics as large as the adenoviral genome, though many issues remain unanswered regarding their functions and relevance in physiological processes. The production and purification of EVs is laborious and their heterogeneity and innate cargo pose a complex problem regarding choosing the correct sources and methods for production and purification. Additionally, understanding and predicting the behavior and cargo delivery of EVs in the target cells is still in its infancy. Regardless of these issues, in studies (I – III) and by others, EVs have been proven to work as targeting carriers of versatile therapeutic cargo, which is in part reflected in the increasing founding and funding of new businesses focused on developing EVs for diagnostic and therapeutic applications. As they resemble naturally occurring liposomes and possess theoretically optimal biocompatibility, while at the same time being non-living, EVs represent a model for the future of drug delivery research. In this thesis, two simple approaches were examined for

preparation of EVs for cancer therapy. The loading of EVs depends on the properties of the therapeutic cargo, but in the studies presented (I – III), loading could be achieved relatively easily by incubating EVs post isolation with hydrophobic PTX, or during EV biogenesis by infecting the secreting cancer cells with the desired oncolytic virus. Both types of therapeutic cargo were successfully delivered into autologous cancer cells with retained or even improved cytotoxicity as was shown with PTX. EVs were primarily internalized by endocytosis, which changed the intracellular distribution of PTX as observed by FLIM for the first time. Additionally, while IEVs were shown to be functioning carriers of oncolytic adenovirus DNA, they are also a novel pathway of spreading viral infection in general. Indeed, as the complex biology behind different kinds of EVs is slowly unraveled, their applicability follows at heel.

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